

POLYPEPTIDES IN PHOTOSYNTHETIC OXYGEN EVOLUTION

Identification of the 33, 23 and 16 kDa proteins

Christer Jansson



Lund 1984



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by

Fil. kand. Christer Jansson (Sm)

AKADEMISK AVHANDLING

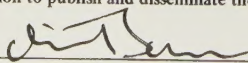
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Abstract Photosynthetic oxygen evolution, carried out by higher plants and algae, is a process of fundamental importance to us and other heterotrophic organisms. It provides the organic compounds and the oxygen required for our cellular energy production. While an impressive amount of kinetic data is available for the process of photosynthetic oxygen evolution, very little is known about the polypeptides involved and about the structure of the enzymatic complex. Photosynthetic oxygen evolution is linked to membrane structures called thylakoids which reside in specialized organelles, the chloroplasts. Recent studies on inside-out thylakoid vesicles have shown that the oxygen evolving complex is located at the inner surface of the thylakoid membrane and have also identified three proteins, at 33, 23 and 16 kDa, as constituents of the oxygen evolving complex. This thesis describes these studies.			
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LIST OF SEPARATE PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals.

- I Trypsination of inside-out chloroplast thylakoid vesicles for localization of the water-splitting site.
Christer Jansson, Bertil Andersson and Hans-Erik Åkerlund (1979) FEBS Lett. 105, 177-180
- II Localization of a 34 000 and a 23 000 M_r polypeptide to the lumenal side of the thylakoid membrane.
Hans-Erik Åkerlund and Christer Jansson (1981) FEBS Lett. 124, 229-232
- III Reconstitution of photosynthetic water splitting in inside-out thylakoid vesicles and identification of a participating polypeptide.
Hans-Erik Åkerlund, Christer Jansson and Bertil Andersson (1982) Biochim. Biophys. Acta 681, 1-10.
- IV Isolation and characterization of a 23 kDa protein essential for photosynthetic oxygen evolution.
Christer Jansson, Hans-Erik Åkerlund and Bertil Andersson (1983) Photosynth. Res. 4, 271-279
- V Proteins involved in photosynthetic oxygen evolution - isolation and characterization.
Christer Jansson (1984) in Proceedings to the 6th International Congress on Photosynthesis (Sybesma, C., ed.) Martinus Nijhoff, The Hague, in press
- VI EPR studies on the photosystem II donor side in salt-washed and reconstituted inside-out thylakoids.
Christer Jansson, Örjan Hansson, Hans-Erik Åkerlund and Lars-Erik Andreåsson (1984) FEBS Lett. submitted
- VII Immunological studies on the organization of proteins in photosynthetic oxygen evolution.
Bertil Andersson, Christer Larsson, Christer Jansson, Ulf Ljungberg and Hans-Erik Åkerlund (1984) Biochim. Biophys. Acta in press



1. INTRODUCTION

Photosynthetic organisms have the ability to utilize solar energy for the reduction of CO_2 to organic compounds, like sugars. For higher plants and algae the electron source for this reduction is water, and the approximate overall reaction is



where $\text{C}_6\text{H}_{12}\text{O}_6$ represents an organic compound. The oxygen produced in this reaction is a biproduct from the oxidation of water and is released to the surroundings. Most heterotrophic organisms, like ourselves, rely on this oxygen and these organic compounds for energy production. During respiration, formally the reverse of photosynthesis, energy is obtained from oxidation of the organic compounds by oxygen. The end product of respiration is CO_2 , which is returned to the surroundings and the photosynthetic organisms.

The use of water as electron source for the photosynthetic production of organic compounds is probably the result of an evolutionary route towards ever more oxidized substrates, perhaps via compounds like NH_2OH and N_2H_2 (1). When finally the ability to use water had evolved, some 3000 milion years ago (1,2), this was an innovation with enormous impact. Until then, there had been no or little oxygen in the atmosphere, and all life had been anaerobic. For these primeval microbes oxygen was highly toxic, and to survive an emerging oxygenic atmosphere they had either to maintain their anaerobic status and escape oxygen, or else they had to develop some metabolic means for protection. One such primitive line of defense was presumably provided by carotenoids (1), but gradually more elaborate mechanisms were evolved. The next logical step in evolution was to learn, not only to cope with the increasing amount of oxygen, but also to benefit from it. This was the starting point for the evolution of cytochrome oxidase

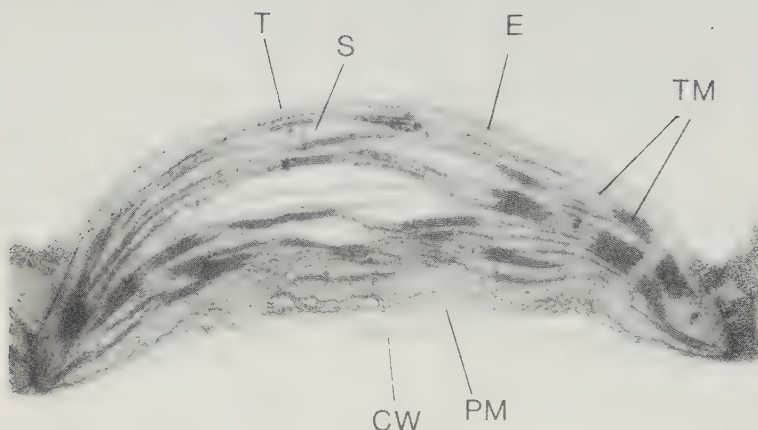


Fig. 1. Electron micrograph of a spinach chloroplast, in situ. PM= plasma membrane; CW=cell wall; E=outer and inner envelope membranes; T=tonoplast; TM=thylakoid membrane; S=stroma. The thylakoid membrane is divided into regions of stacked membrane vesicles, grana, interconnected by a network of stroma lamellae. The internal space of the grana and stroma lamellae is continuous. (By courtesy of Drs Christer Larsson and Curt Collin.)

and the respiring organisms. The acquisition of oxygenic photosynthesis was a very successful step; water is a common and plentiful compound. The primeval microbes that first began to evolve oxygen were the forerunners of present-day cyanobacteria and prochlorophyta, and of the

chloroplasts in green plants and algae.

Photosynthesis in most organisms consists mainly of two phases. The first phase is the photosynthetic electron transport, where light is harvested by chlorophyll and converted to the energy-rich compound ATP and reducing agents like NADPH. In the second phase, the Calvin cycle, CO_2 is fixed and reduced to organic compounds in an energy-consuming process requiring the ATP and NADPH. In green plants and algae, these photosynthetic reactions are carried out in special organelles, the chloroplasts (Fig. 1). The photosynthetic electron pathway resides in the thylakoid membrane, while the enzymes of the Calvin cycle are located in the stroma, the soluble part between the thylakoid and the envelope.

The general features of electron transport in oxygenic photosynthesis are illustrated by the Z scheme of Fig. 2. There are two photosystems, photosystem I and photosystem II, acting in sequence. Each photosystem contains reaction center chlorophyll which is connected to a large number of antenna chlorophyll. The reaction center chlorophyll of photosystem I and II is called P680 and P700 respectively. By absorbing a quantum of light, or receiving it by excitation transfer from the antenna chlorophyll, P680 and P700 are promoted to an excited state. The excited P680 and P700 donate one electron each to their respective electron acceptors, and the oxidized P680 and P700 are subsequently re-reduced by electron donors, and can again be excited by a new quantum of light. The ultimate electron acceptor in this electron transport chain is CO_2 and the ultimate electron donor is H_2O .

The photosynthetic oxygen evolution is linked to photosystem II with the following overall stoichiometry



The redox potential for this reaction is 800 mV and a very

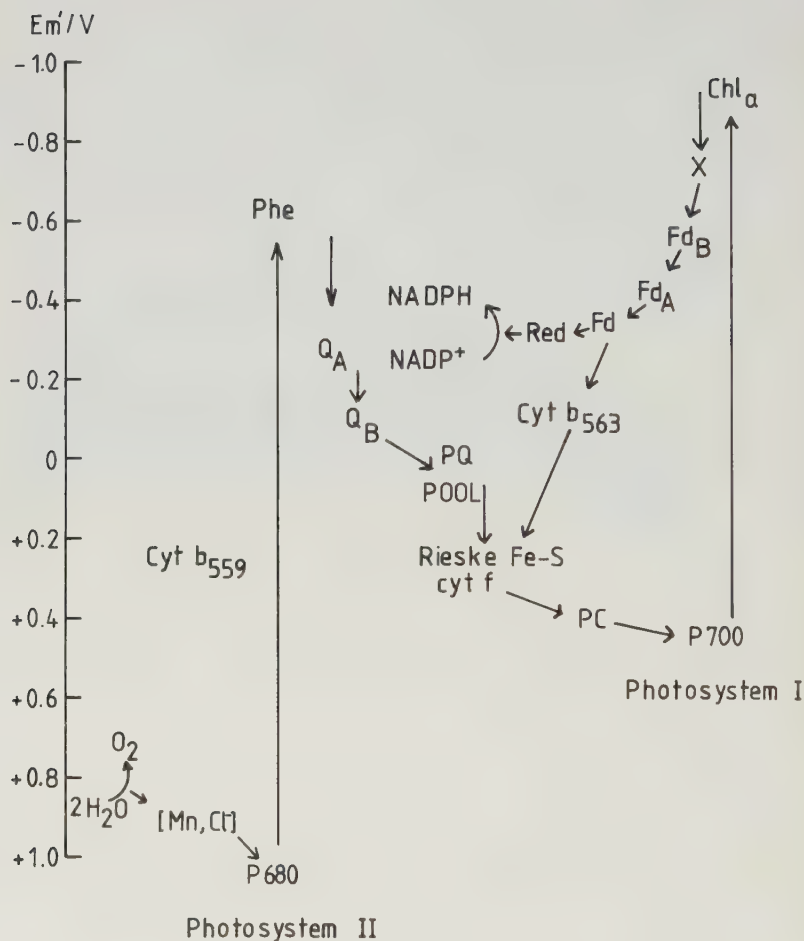


Fig. 2. The photosynthetic electron transport, the so-called Z scheme. P700 and P680=the reaction center chlorophyll of photosystem I and II respectively; Z=a plastoquinol, the immediate donor to P680; Phe=pheophytin, the immediate acceptor to P680; Q_A and Q_B =quinones; PQ=plastoquinone; PC=plastocyanin; chl a =chlorophyll a , the immediate acceptor to P700; Fd_A and Fd_B =bound ferredoxin; Fd=soluble ferredoxin; Fe-S=iron-sulphur center; cyt=cytochrome; X=unknown component; Red=Fd-NADP oxido-reductase; E_m' =the redox potential at pH 7.

strong oxidant is required to extract the four electrons from water. This strong oxidant is $P680^+$, the photo-oxidized reaction center chlorophyll of photosystem II. However, while the oxidation of water involves four electrons, P680 can only extract one electron at the time. Therefore, an interfacing system is required to connect these one-electron and four-electron processes. The system selected by Nature to accomplish this task, was not a cooperation between several P680, but a sophisticated mechanism where water is oxidized in consecutive steps and the highly reactive oxidation intermediates are stabilized. Possibly, this mechanism was a modification of a previous system, used for the oxidation of some nitrogen compound. The enzymatic machinery responsible for the photosynthetic oxidation of water is designated the oxygen evolving complex.

Although an impressive amount of kinetic data has accumulated for the oxygen evolving process, information about the polypeptides involved is lagging markedly behind. However, in the last years, exciting progress has been made, mainly through the intensive studies on three proteins of approximately 33, 23 and 16 kDa. This thesis focuses on the identification, isolation and investigations of these three proteins.

Before dealing with the 33, 23 and 16 kDa proteins, a general treatment of the mechanism of photosynthetic oxygen evolution is required. The two following sections deal briefly with the basic features of the S-cycle hypothesis and catalogue the cofactors implicated in the oxygen evolving process. Section 4. is a discussion of cytochrome b_{559} and its possible function during oxygen evolution. The fifth and major section is devoted to the 33, 23 and 16 kDa proteins.

2. The S-cycle

With the improved oxygen electrode of Joliot (3), it was demonstrated in the 1960s by Joliot and coworkers (4-6) that during illumination of dark-adapted *Chlorella* with a train of flashes, the oxygen yield was low on the first two flashes, maximal on the third and then followed by a damped oscillation with a periodicity of four. To account for these data, and for results of their own, Kok et al. (7), in 1970, proposed a hypothesis that has been known as the S-cycle (Fig. 3). The S-cycle has been a very successful formalistic model of the oxygen evolving process.

The S-cycle mechanism for oxygen evolution proposes five states, S_0 through S_4 . These states represent consecutive stages of oxidation within the oxygen evolving complex. Following a photoact by photosystem II, S_n advances to S_{n+1} . S_4 is unstable and reverts spontaneously to S_0 , during release of oxygen. In dark-adapted thylakoids only S_0 and S_1 are stable, with a distribution that follows $S_1/S_0=3$. S_2 and S_3 relax in the dark to S_1 with a half time of 40-60 s (8). This would explain why, in dark-adapted thylakoids, the maximum oxygen yield appears on the third flash.

Fig. 3 is a summarizing scheme of the reactions on the donor side of photosystem II. It is implicit in the S-cycle hypothesis that there is no cooperation between different photosystem II units, but each oxygen evolving complex is uniquely connected only to one P680. The species Z, probably a bound plastoquinol (9), is EPR detectable in its oxidized state (the cation radical), giving rise to Signal II_{vf} (10-12). Thus, the decay kinetics for Signal II_{vf} following a flash, reflects the reduction of Z^+ by the various S-states (13). However, no conclusive evidence has yet been presented for a reduction of Z^+ by S_0 and S_1 (14). In an alternative model proposed by Bouges-Bocquet (15) there are two intermediate do-

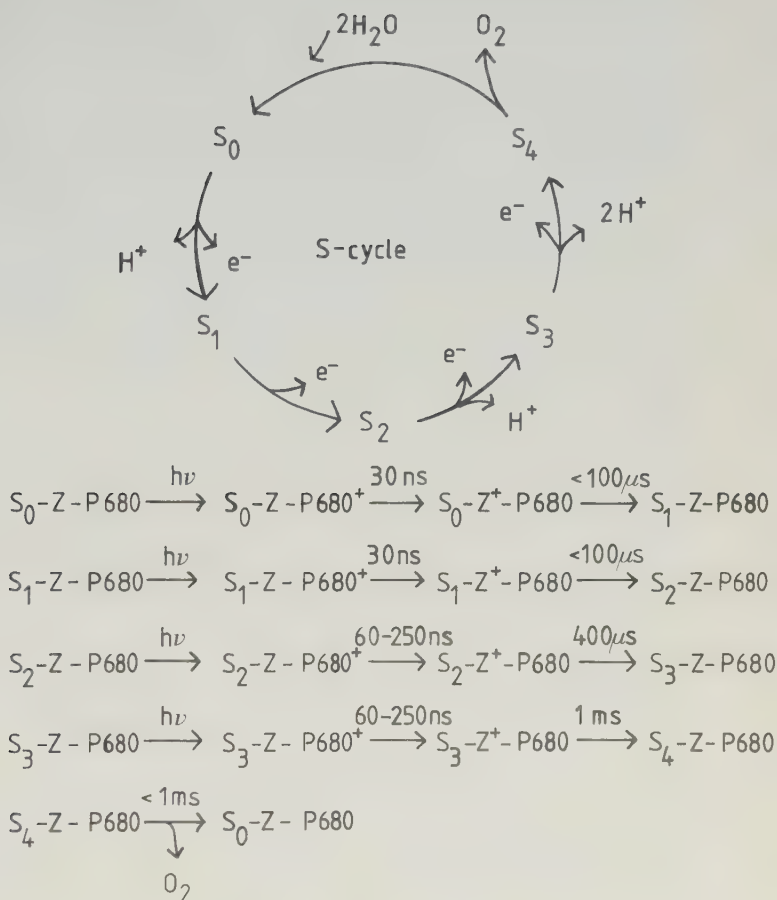


Fig. 3. The S-cycle hypothesis showing the cycling of the water-oxidizing site through five different oxidation states, S_0 , S_1 , S_2 , S_3 , S_4 (16).

Following absorption of a quantum of light, the generated $P680^+$ is reduced by the donor Z. Z^+ is subsequently reduced by the water-oxidizing site. The various times (16-18) refer to half times.

nors, Z_1 and Z_2 . Z_1 is reduced by S_0 and S_1 while Z_2 is reduced by S_2 and S_3 . The oxidation of S_0 and S_1 proceeds with a half time of less than 100 μ s (13) and values of 3 and 2 μ s, respectively, have been suggested (15). The oxidation of S_2 and S_3 is considerably slower. Under repetitive flash illumination the half times for the S-transitions are averaged to around 700 μ s (13). The midpoint potential for the water-oxidizing reaction at pH 7 is 800 mV. Thus, the average midpoint potential for the $S_0 \rightarrow S_4$ transitions must be >800 mV. The potential for the four individual steps have been estimated to approximately 240, 890, 930 and 870 mV for the $S_0 \rightarrow S_1$, $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4$ transitions, respectively (15).

The stoichiometric release of protons during the S-transitions is controversial and different patterns have been suggested (19,20). The 1,0,1,2 pattern depicted in Fig. 3 is supported by several experimental findings (19-23). All recent results on proton release during the water oxidation process exclude a model where all four protons are released with oxygen in a final concerted reaction. This implies that the various S-states involve water and its oxidation intermediates. Evidently, since not only electrons but also protons are released in a sequential manner during the S-cycle, the notion of the oxygen evolving complex as a system able to accumulate four positive charges is misleading.

3. COFACTORS

3.1. Manganese.

Several models have been proposed for the molecular substantiation of the S-cycle (15, 24-28). The corner stone in these models is a coordinated manganese cluster, that undergoes redox changes during advancement of the S-states. Water and its oxidation intermediates are bound as ligands to the manganese cluster. Manganese has long been attractive in this function because of its multiple oxidation states, some of which involve high redox potentials also in complexed forms (29). There is also strong experimental support for this implication since EPR (24) and NMR (30) measurements have demonstrated periodic changes in the oxidation states of manganese, in synchrony with the oscillation of oxygen evolution. A multiline EPR signal reported by Dismukes et al. (31-32) has been shown to arise from the S_2 -state (8,31,33) and ascribed to a manganese dimer or tetramer (26,33,34). Furthermore, recent characterization of absorbance changes in the ultraviolet region (14) suggests that the $S_1 \rightarrow S_2$ transition involves the oxidation of Mn^{3+} to Mn^{4+} .

There is a large controversy as to the number of manganese associated with water oxidation, and both bi- (15,24,28) and tetra-(26) nuclear manganese clusters have been suggested. Although most studies agree on a minimum number of four manganese per P680 (35-39), there seems to be a functional (35,37) as well as spatial (36) heterogeneity in this pool of manganese. An oxygen evolving photosystem II preparation with only two manganese per P680 has also been described (40,41).

3.2. Chloride.

The requirement for Cl^- as a cofactor in oxygen evolution was reported by Warburg and Lüttgens already in 1944 (42), but has long been overlooked. Although its require-

ment is well established (43-45), the precise function of Cl^- is not obvious. Several indications point to an interaction between Cl^- and the manganese cluster (45-49). From the work of Critchley et al. (45), Govindjee et al. (49) and Izawa et al. (48) it can be inferred that binding of Cl^- to the manganese cluster is necessary for at least the $\text{S}_1 \rightarrow \text{S}_2$ transition. In a tentative mechanism proposed by Govindjee et al. (49) it is suggested that successive binding and unbinding of Cl^- facilitates the release of protons. Sandusky and Yocum (50) concluded that Cl^- binds as a ligand between two manganese or between manganese and the species Z, or both, and functions by mediating electron transfer. Cl^- has also been suggested merely as a counter-ion to positively charged S-states (24). Also a structural role for Cl^- was considered by Critchley (51). She suggested that the binding of Cl^- induces conformational changes that stabilize the oxygen evolving complex. Possibly, Cl^- can serve in several of these functions, consistent with the findings of two pools of bound Cl^- associated with oxygen evolution (49). The number of binding sites for Cl^- in the oxygen evolving complex is controversial and values between 5 and 37 have been reported (44,52).

3.3. Calcium

The function of Ca^{2+} in the oxygen evolving process is probably structural, and it is likely to have a stabilizing effect on the oxygen evolving complex. During photoactivation of oxygen evolution, Ca^{2+} was required for a tight incorporation of manganese in the oxygen evolving complex (53,54), and photoreactivation of oxygen evolution was dependent on Ca^{2+} for maximal restoration (55-57). Ca^{2+} was also shown to be essential for retention of certain polypeptides in photosystem II preparations from cyanobacteria (58), and to influence the polarity around the species Z (59). Very recently, Ca^{2+} was also found to prevent photoinhibition (60). These studies have suggested a specific binding site for

Ca^{2+} in the oxygen evolving complex, and from experiments with calmodulin antagonists (53,54,61,62) the presence of calmodulin or a calmodulin-like protein has been inferred. A 16-17 kDa protein has also been isolated from chloroplasts and shown to stimulate oxygen evolution in the presence of Ca^{2+} (62). However, the functional site for this protein was probably on the acceptor side of photosystem II.

4. CYTOCHROME b_{559}

There may be two distinct species of cytochrome b_{559} in the thylakoid membrane (63-65). A low-potential form with a midpoint potential of around 60 mV in the stroma lamellae, and a high-potential form with a midpoint potential of around 375 mV in the partition region, the appressed part of the grana. The role of cytochrome b_{559} in photosynthesis is not known. However, there is an impressive amount of circumstantial evidence that links the high-potential form to the donor side of photosystem II (65). Treatments that inhibit oxygen evolution also lower the potential of this cytochrome from 375 mV to a variety of lower potential forms (66). Mutants with lowered potential of cytochrome b_{559} were also deficient in oxygen evolution (67-69). Revertants with restored oxygen evolution recovered the high-potential form (69). Moreover, from these inhibitory and mutant studies, a close correlation was found between the high-potential form of cytochrome b_{559} and the content of manganese. It was suggested (65) that the release of manganese indirectly, through conformational changes, lowers the potential of cytochrome b_{559} .

Photoreduction of P680 by cytochrome b_{559} has been observed (65,70,71), when inhibition of the oxygen evolution has prevented electron transfer from the water-oxidizing site. This has led to speculations (65,70) that one function of cytochrome b_{559} is to keep P680 reduced in events of deactivation of the oxygen evolving complex. Since $P680^+$ is a very strong oxidant, such a safety mechanism would prevent deleterious photo-oxidation of pigments and other lipids. The reductant for cytochrome b_{559} in these situations could be ascorbate (65), which is present in high concentrations in the stroma (72).

According to the hypothesis of Butler (73), cytochrome b_{559} has a dual function. The catalytic cycle of the

cytochrome is assumed to involve a change in both redox state and midpoint potential. During this cycle, cytochrome b_{559} would reduce cytochrome f or plastocyanin on the acceptor side of photosystem II, and serve on the donor side as a proton translocator in at least the $S_0 \rightarrow S_1$ transition. The low midpoint potential for the $S_0 \rightarrow S_1$ transition (Section 2.) has also led others to consider the involvement of cytochrome b_{559} in this step (25).

Another possible function for cytochrome b_{559} is in a cyclic electron flow around photosystem II (70,74,75). This cycle might be turned on and off as a response to the levels of CO_2 , ATP or NADP (75), or to phosphorylation of thylakoid polypeptides (76,77).

The amount of the high-potential form of cytochrome b_{559} per P680 has been estimated to one (69, 78-80) or two (81). The apparent molecular weight of the cytochrome on SDS-gels is around 10 000 (82), and it probably consists of one or two polypeptide chains per heme (82).

5. THE 33,23 AND 16 kDa PROTEINS

5.1. Perspectives and identification

Identification of polypeptides involved in photosynthetic oxygen evolution has, until recently, met with limited success. Apart from the studies on cytochrome b_{559} (Section 4.) the only evidence of any significance has been the results from mutational analyses by Bishop and coworkers (67,68). From these studies a 34 kDa polypeptide was inferred as a manganese-binding protein (Section 5.6.2.). The results reported by Spector and Winget (83,84) of a 65 kDa manganese protein, and by Nakatani and Barber (85,86) of a 59 kDa catalase protein, remain to be verified. The nature of the 13 kDa manganese protein with catalase activity, isolated from cyanobacteria, (87,88) is still an open question. Some other reports (89,90) of manganese-binding proteins can probably be ascribed to contaminating manganese. Early, preliminary studies (91-93) on isolated proteins reported to stimulate oxygen evolution have not been extended.

This slow progress is mainly due to the shielded location of the oxygen evolving complex at the inner thylakoid surface (I-III,VI,VII). It has been difficult to affect and study the oxygen evolving complex without deleterious treatments. However, with the introduction of inside-out thylakoid vesicles (94,95), and later with various oxygen evolving photosystem II detergent preparations (78,79,96-98), a new and intensive era started in the study of photosynthetic oxygen evolution. In the inside-out thylakoids the inner thylakoid surface is exposed to the external medium. The mechanism for their formation has been outlined by Andersson et al. (99,100), as illustrated in Fig. 4. The inside-out thylakoids are derived from the partition region and are highly enriched in photosystem II. In the photosystem II detergent preparations, the membrane fragments have the same origin and everted configuration as the inside-out thylakoids (102,103).

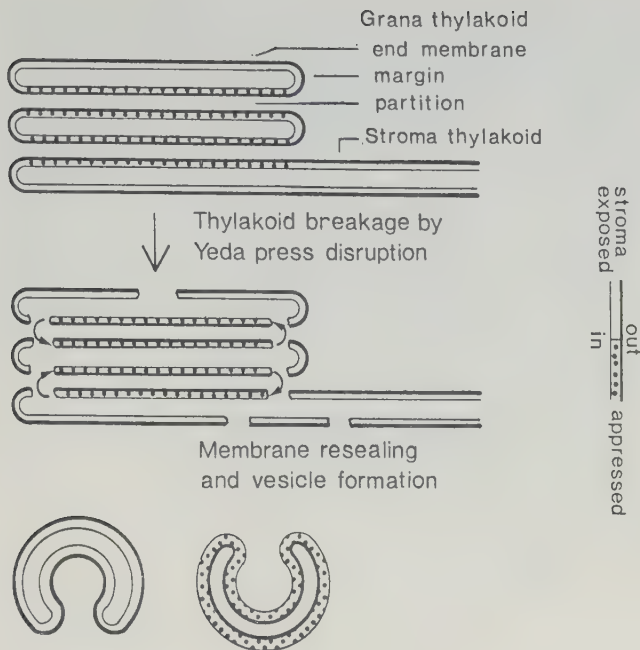


Fig. 4. Proposed mechanism for the formation of inside-out thylakoid vesicles (101-103). Following Yeda press disintegration, the inside-out vesicles are separated from right-side out material by partition in an aqueous dextran/poly(ethylene glycol) two-phase system (101).

However, there are two notable features distinguishing these two preparations;

- 1). The inside-out thylakoids are prepared by phase-partition following mechanical disruption. Thus, they are obtained without the use of detergents that may interfere with the structural integrity of the membrane (104).
- 2). The inside-out thylakoids are closed membrane vesicles. In contrast, the detergent preparations mostly consist of unsealed pairs of membranes, stabilized at the edges by the detergent (102,103).

Therefore, in studies on the organization of the thylakoid membrane, the inside-out thylakoids are an outstanding material. On the other hand, when viewed as photosystem II preparations, the simple polypeptide pattern and the high yield for many detergent preparations, make them highly advantageous.

The trypsination study on inside-out thylakoids in paper I was the first direct approach to examine the transverse location of the water-oxidizing site in the thylakoid membrane. This issue was earlier controversial, and circumstantial evidence for both an internal (105,106) and external (107-109) location had been reported. The results in paper I clearly demonstrate the susceptibility of the oxygen evolving activity to tryptic digestion in the inside-out thylakoids. In contrast, the DPC-mediated electron transport was not inhibited. A comparison between the effects on inside-out and right-side out thylakoids, strongly suggests that the major part of the oxygen evolving complex, including the water-oxidizing site, is located at the inner thylakoid surface. Since the donor site for DPC is the species Z (110), the results further suggest a buried position for the rest of the donor side in photosystem II. An internal location of the water-oxidizing site does not exclude that it can be indirectly affected from the outer thylakoid surface. In fact, the results in paper I suggest such a possibility.

Subsequent studies on inside-out thylakoids confirmed the internal location of the oxygen evolving complex (I-III). Original observations from these studies, led to the recognition of three polypeptides, located at the inner thylakoid surface, as likely constituents of the oxygen evolving complex;

- 1). Mild tryptic digestion, which specifically inhibited the oxygen evolving activity in inside-out thylakoids (I), degraded a 34 kDa polypeptide in inside-out but not in right-side out thylakoids (II). These results were particularly interesting in relation to the reports from

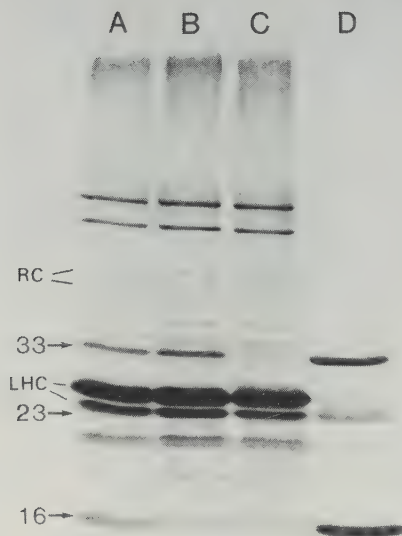


Fig. 5. Polypeptide pattern for untreated inside-out thylakoids (A), NaCl-washed inside-out thylakoids (B), Tris-washed inside-out thylakoids (C) and a mixture of the isolated 33, 23 and 16 kDa proteins (D). RC=the 47 and 43 kDa reaction center polypeptides of photosystem II; LHC=the 28-24 kDa polypeptides of the light-harvesting complex of photosystem II. In the membrane fractions, the 23 kDa protein is poorly resolved from LHC.

Metz et al. (67) and Metz and Bishop (68) on a 34 kDa polypeptide missing in algal mutants with impaired oxygen evolution, and from Kuwabara and Murata (111) on the isolation of a 33 kDa protein enriched in photosystem II preparations.

2). Washing with alkaline Tris, a potent inhibitor of oxygen evolution (38,98,112-114), released a 34 and 23 kDa polypeptide in inside-out but not in right-side out thylakoids (II). Similar results were later reported also for photosystem II detergent preparations (96).

3). Washing with high concentrations of NaCl inhibited the oxygen evolution only in inside-out thylakoids, concomitant with a release of a 23 and a 16 kDa polypeptide (115,III).

Hereafter these three polypeptides will be referred to as the 33, 23 and 16 kDa proteins (Fig. 5).

The original observation by Åkerlund (115), that NaCl treatment inhibited oxygen evolution in inside-out but not in right-side out thylakoids, suggested reversible release of proteins from the inner thylakoid surface, as illustrated in Fig. 6. In right-side out thylakoids, the released components were trapped in the thylakoid lumen and therefore able to rebind under low ionic conditions. In contrast, for inside-out thylakoids they were lost to the external medium. This argued for reconstitution experiments (115,III) with proteins released from inside-out thylakoids. In paper III it is demonstrated that the lost oxygen evolution, following NaCl treatment of inside-out thylakoids, could indeed be restored by addition of the released 23 and 16 kDa proteins under low ionic conditions. It is further evident that the added proteins had actually rebound to the membrane. The addition of the 23 and 16 kDa proteins to right-side out thylakoids or to untreated inside-out thylakoids had no effect. To examine the restorative effect for each of the 23 and 16 kDa proteins, a purification procedure was developed (Section 5.2.). With the purified proteins preparations we could demonstrate (III,IV) that the 23 kDa protein was respon-

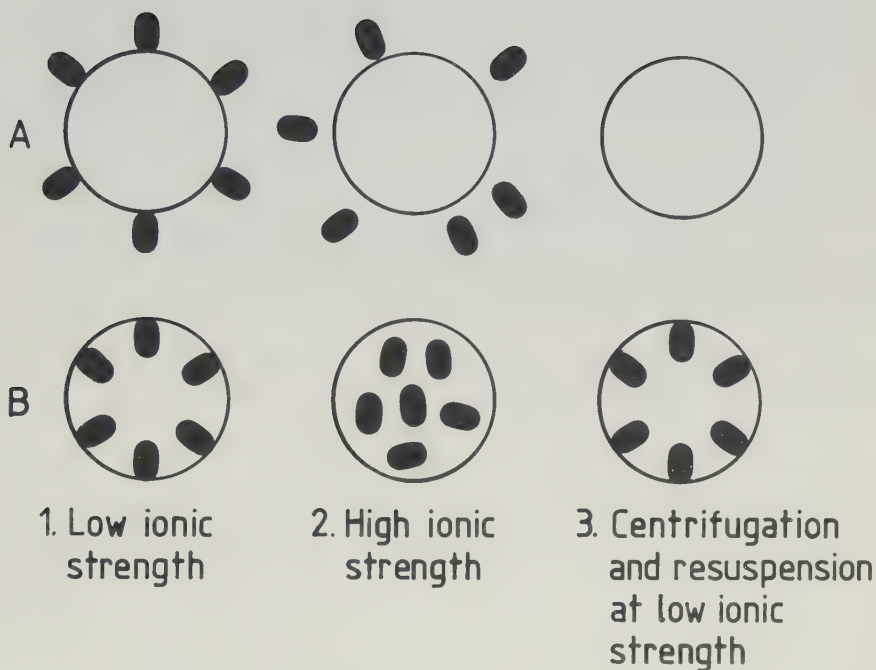


Fig. 6. Schematic representation of release and reassociation of proteins after salt washing of inside-out (A) and right-side out (B) thylakoids.

sible for the restoration of the oxygen evolution. In contrast, the 16 kDa protein exhibited only a small stimulatory capacity which was ascribed to contamination by the 23 kDa protein (III). This conclusion was later supported in a more thorough investigation by Ljungberg et al. (116). That the functional site for the 23 kDa protein is indeed on the donor side of P680 was demonstrated in paper III by fluorescence analyses and later in paper VI by studies on Signal II_F. This was also confirmed by Akerlund

et al. (117) from analyses on the decay kinetics for P680 reduction.

The reconstitution work in paper III was the first clear-cut evidence for the involvement of the 23 kDa protein on the donor side of photosystem II. These further functional studies on the 23 kDa protein will be considered in detail in Section 5.6.1. As for the 33 and 16 kDa proteins, several additional evidence for their involvement in the oxygen evolving process have been obtained, and the functional aspects for these proteins will be discussed in Sections 5.6.2. and 5.6.3.

5.2. Purification

To obtain the purified 33, 23 and 16 kDa proteins in amounts sufficient for characterization and reconstitution studies, a procedure via Tris or NaCl washing of inside-out thylakoids was found rather cumbersome. Therefore, an alternative approach was chosen. Since the 33, 23 and 16 kDa proteins are all water soluble, a crude protein extract containing these proteins was obtained by water extraction of delipidated thylakoid membranes (III,IV,V). Using this crude protein extract as starting material, a combination of ion exchange and affinity chromatography yielded highly purified preparations for each of the 33, 23 and 16 kDa proteins (IV,V). The yield, based on the crude protein extract, was 25-30% for each of the proteins. Purification procedures for the 33, 23 and 16 kDa proteins have also been described by Yamamoto et al. (118), Kuwabara and Murata (119), Lindberg-Møller and Høj (103) and Fukutaka et al. (120).

5.3. Characterization

The characterization of the isolated 33, 23 and 16 kDa proteins is summarized in paper V. The physico-chemical properties of the proteins give no hints as to their function in the thylakoid membrane. Neither of them contains any metals and they exhibit no significant absorbance in the visible region of the spectrum. Although this argues against any of the proteins being a redox component, this possibility can not be entirely excluded (IV). The amino acid analysis shows that all three proteins are highly hydrophillic, with polarity indexes between 51 and 48%. This is consistent with their release from the thylakoid membrane and their solubility in water without the presence of detergents (II,III,VII), and suggests that they are extrinsically bound at the inner thylakoid surface. The content of lysine + arginine is quite high for all three proteins, although most prominent for the 16 kDa protein. The isoelectric points are 5.2, 7.3 and 8.5 for the 33, 23

and 16 kDa proteins respectively. Both the 23 and 16 kDa proteins contain only one cysteine residue, and the 16 kDa protein also lack methionine. The 33 kDa protein lacks histidine.

To the data in in paper V can be added that the molecular weight estimated by gel filtration was 32 000, 25 000 and 23 000 for the 33, 23 and 16 kDa proteins respectively, in the presence of 100 mM NaCl at pH 7.3 (Jansson, C. unpublished observation) This high molecular weight for the 16 kDa protein was noted also by Kuwabara and Murata (119). It probably reflects a non-globular shape of the protein under the conditions employed.

The reason for the fuzzy appearance of the 23 kDa protein on SDS-gels (III-V) is not yet known. One possible explanation is that the single cysteine residue is difficult to maintain reduced during electrophoresis. Variability in the electrophoretic mobility for the 23 kDa protein has also been observed after alkaline Tris treatment (Kuwabara, T., personal communication) and after treatment with NaCl at alkaline pH (103.) A shift in the apparent molecular weight was reported upon exchanging SDS for LDS during solubilization (38).

The amino acid analyses for the 33, 23 and 16 kDa proteins in paper V resemble very much those of Kuwabara and Murata (119) but show some marked deviations from those of Yamamoto et al. (118). The 24 kDa protein described by Yamamoto et al. has, in comparison, a remarkably high content of arginine. From the densitograms it is not possible to evaluate the purity of the fractions analyzed by Yamamoto et al. and their 24 kDa preparation might have been contaminated. Their 24 and 18 kDa preparations probably also contained nucleic acids, or quinones, as judged from their absorbance maxima at 266 nm. However, considering their general characteristics, the three proteins reported by Yamamoto et al. most certainly are the same as those described in paper V.

5.4. Distribution among species

The presence of the 33, 23 and 16 kDa proteins in species other than spinach can be deduced from a variety of studies. Antibodies against the spinach 33, 23 and 16 kDa proteins have been raised (VII) and used in immunological studies (VII,121-124). From such studies the 33, 23 and 16 kDa proteins have been found by Westhoff in *Oenothera* (Westhoff, P., personal communication), and by Andersson et al. (121) in mangrove. Their presence in pea is indicated from biochemical studies (125). Despite the scarcity in data, it can be expected that the 33, 23 and 16 kDa proteins are ubiquitous among dicots. Immunological studies revealed the presence of the 33 and 23 kDa proteins in barley and maize (Westhoff, P., personal communication). The amino acid analysis presented by Lindberg-Møller and Høj (103), of proteins released by Tris/NaCl treatment of photosystem II detergent preparations from barley, showed a 32 and a 23 kDa protein rather similar to the 33 and 23 kDa proteins from spinach (V), although the isoelectric point for the barley 23 kDa protein was as low as 5.1 compared to 7.3-6.4 for spinach (IV,V,118,119). Interestingly enough, no 16 kDa protein was found in this study. A basic 13.5 kDa protein was released together with the 32 and 23 kDa proteins, but any relation to the spinach 16 kDa protein is difficult to establish from the amino acid compositions and has to await immunological studies. Cammarata et al.(38) reported all three proteins in photosystem II detergent preparations from wheat. Apart from the angiosperms mentioned above, antibodies against the spinach 33 kDa protein was found to cross-react with species from such diverse groups of plants as gymnosperms and ferns (Westhoff, P., personal communication). The 33, 23 and 16 kDa proteins have also been immunologically identified in *Chlamydomonas* (Andersson, B. personal communication) and indicated from mutational analyses also in *Scenedesmus* (69). Preliminary studies with the antibodies against the spinach proteins showed no cross reaction with cyanobacteria or prochlorophyta

(Andersson, B., personal communication). The polypeptide patterns on SDS-gels of oxygen evolving photosystem II preparations from *Phormidium laminosum* (126) show several polypeptides in the 34-30 and 18-15 kDa region, but none in the 25-20 kDa region.

5.5. Stoichiometry

The stoichiometric amounts of the 33, 23 and 16 kDa proteins in the thylakoid membrane was investigated in paper VII, employing rocket immunoelectrophoresis. This showed a protein/chlorophyll ratio for intact thylakoids of around 1/200 for each of the three proteins. Assuming a Q/chlorophyll ratio of 1/300 for intact thylakoids, according to Anderson and Melis (127), this yields 1-2 copies of each protein per photosystem II unit. However, Andersson and Haehnel (128) determined the same Q/chlorophyll ratio to 1/500. This would yield 2-3 copies of each protein per photosystem II unit. The studies of Anderson and Melis, and of Andersson and Haehnel were both based on the light-induced absorbance changes of Q. A quite independent determination of the unit size of photosystem II is obtained from the results in paper VI, where it is shown that the amplitude of Tris-induced Signal II_f corresponded to 1 spin per 250 chlorophyll for the inside-out thylakoids. In the studies of Anderson and Melis and of Andersson and Haehnel the Q/chlorophyll ratio for inside-out thylakoid preparations were 1/273 and 1/380, respectively. Thus, the quantification in paper VI supports the work of Anderson and Melis, and a number of 1-2 copies of the 33, 23 and 16 kDa proteins per photosystem II unit. In paper III the stoichiometry for the 23 kDa protein was calculated from the stimulation curve. Adopting the value from paper VI of 250 chlorophyll per photosystem II, a recalculation of the results in paper III yields 1 copy of the 23 kDa protein per photosystem II unit. Based on the intensity of coomassie-stained gels, Murata and coworkers (39) concluded one copy of each protein per photosystem II unit, while Cammarata et al. (38) reported two for each of the 33 and 23

kDa proteins. Thus, while the molar ratio of 1:1:1 for the 33, 23 and 16 kDa proteins seems clear, the question whether there are one or two copies per photosystem II remains to be solved. Pertinent to these aspects is the correlation obtained in paper VII, between the release of the 23 kDa protein from inside-out thylakoids upon NaCl washing and the inhibition of oxygen evolution. These results suggest that if there are two copies of the 23 kDa protein per photosystem II, both copies were released simultaneously from the membrane.

The 33, 23 and 16 kDa proteins are all highly enriched in the partition region (VII,129, Andersson, B., Klein-Hitpass, L., Jansson, C. and Berzborn, R., unpublished observation). From the lateral index established by Andersson (130), it is evident that, on a chlorophyll basis, there are around seven times higher concentration of the 33, 23 and 16 kDa proteins in the grana compared to the stroma lamellae. Roughly the same lateral distribution was observed also for other photosystem II polypeptides and for the oxygen evolving activity (129).

5.6. Function

5.6.1. The 23 kDa protein

In order to pin-point the functional site of the 23 kDa protein on the donor side of photosystem II, the influence of the 23 kDa protein on the amplitude of Signal II_f was studied (VI). Signal II_f is the EPR signal arising from Z^+ in photosystem II units where the electron transport between the water-oxidizing site and Z has been inhibited (130). The results in paper VI demonstrate that the release of the 23 kDa protein from inside-out thylakoids increased the amplitude of Signal II_f . Upon readdition of the 23 kDa protein the amplitude again decreased. This implies that the 23 kDa protein influences the electron transport between the water-oxidizing site and Z. Since Z is inferred to be closely associated with the manganese cluster (36), paper VI provides strong support for a func-

tional site of the 23 kDa protein in close proximity to the actual water-oxidizing site. Compared to the degree of inhibition of oxygen evolution, the increase in Signal II_f induced by NaCl treatment of the inside-out thylakoids was rather small (VI). This suggests that in most of the inhibited photosystem II units Z^+ is still rapidly reduced, with a half time <30 ms, the response time of the experimental set-up. The electron source(s) for this rapid donation might have been endogenous or exogenous. The hypothesis favoured in paper VI is, that in a substantial part of the inhibited photosystem II units one or some of the S-transitions are still operative in a cyclic mechanism, thereby reducing Z^+ . Thus, it is predicted, that with a better time resolution a poly-phasic decay of Signal II_f would have been obtained after NaCl treatment. This interpretation is supported by results from Wensink and van Gorkum (131). They concluded that in NaCl-treated photosystem II detergent preparations the $S_1 \rightarrow S_2$ transition was unaffected while the $S_3 \rightarrow S_4$ transition was blocked. The results in paper VI also show that, in contrast to NaCl treatment, Tris treatment of the inside-out thylakoids induced a large increase in Signal II_f . This reflects the quite different actions of these two treatments. NaCl treatment does not release the 33 kDa protein (115,III) nor Mn (116). Neither does it seem to affect the structural integrity of the manganese cluster (132). Alkaline Tris, on the other hand, readily releases both the 33 kDa protein and manganese (38,39,133). The demonstration of a reversible increase in Signal II_f after release of the 23 kDa protein is in contrast to the results of Wensink and van Gorkum (131). They observed no increase in Signal II_f in NaCl-treated photosystem II detergent preparations.

Ghanotakis et al. (132,134) and Miyao and Murata (135) recently demonstrated that the lost oxygen evolution in NaCl-washed photosystem II detergent preparations was largely restored by addition of Ca^{2+} , but not of other divalent cations. It was also found (134) that addition of

the 23 and 16 kDa proteins to the depleted membranes increased the affinity for Ca^{2+} . Moreover, removal of loosely bound Ca^{2+} prior to the addition of the proteins, abolished the restoration of oxygen evolution. Simultaneously, Andersson et al. (121) showed that oxygen evolution could be restored in NaCl-washed inside-out thylakoids by addition of Cl^- , and that the 23 kDa protein increased the affinity for Cl^- . Nakatani (60) demonstrated the requirement for both Cl^- and Ca^{2+} in the restoration of oxygen evolution in NaCl-washed detergent preparations. The function for Cl^- in oxygen evolution is probably associated with the S-cycle, while a structural role is likely for Ca^{2+} . Thus, it can be envisaged that the presence of Ca^{2+} imposes conformational stability on the water-oxidizing site, which may also facilitate the binding of Cl^- . This would be consistent with the stabilizing effects previously observed with Ca^{2+} (Section 3.3.). It is tempting to speculate that, while the 23 kDa protein increases the affinity for Cl^- , the 16 kDa protein is involved in the binding of Ca^{2+} . This may be supported by the observation that no complete restoration of oxygen evolution has been obtained by addition of only the 23 kDa protein to NaCl-washed membranes, while Miyao and Murata (136) obtained complete restoration with a combination of the 23 and 16 kDa proteins.

Considering the results of Andersson et al. (121) it can be speculated that the relatively small increase in Signal II_f observed in paper VI, after NaCl treatment of inside-out thylakoids, might to some extent be due to the rather high concentrations of Cl^- used in these measurements. Thus, it is now urgent to repeat the EPR studies under varying Cl^- conditions. This should give valuable information about the behaviour of Cl^- at the water-oxidizing site, in the presence and absence of the 23 kDa protein.

5.6.2. The 33 kDa protein

In the search for the manganese-binding protein(s) of the oxygen evolving complex, the 33 kDa protein has been an attractive candidate. However, the correlation between the 33 kDa protein and manganese is ambiguous. The isolated 33 kDa protein does not contain manganese (IV), but since higher oxidation states of manganese may readily be reduced during protein isolation, and Mn^{2+} -proteins are very unstable (137), this is no conclusive information. Recently, Dismukes et al. (34) described the purification of a manganese-containing 34 kDa protein from thylakoid membranes, using oxidizing conditions. This protein was contained in an unpure fraction and, therefore, no definite conclusions can be made at present. Various alkaline treatments (38,39,133,138, Andersson, B., Klein-Hitpass, L., Jansson, C. and Berzborn, R., unpublished observation) and treatments with urea (39) readily released both the 33 kDa protein and manganese. On the other hand, treatments with low concentrations of NH_2OH at neutral pH (39) resulted in a substantial loss of manganese without affecting the 33 kDa protein, and under certain conditions it was possible to release the 33 kDa protein without significant losses of manganese (114,133,139,140). Algal (67-69) and maize (141) mutants deficient in oxygen evolution and with a low content of manganese, lacked a 34-32 kDa polypeptide. When revertants were developed from mutants lacking the 34-32 kDa polypeptide and manganese, the restoration in oxygen evolution was accompanied by a return of both the 34-32 kDa polypeptide and manganese (69). These results argue strongly for the involvement of a 34-32 kDa polypeptide in the binding of manganese and support the notion of the 33 kDa protein as a manganese protein. However, identification of polypeptides based merely on their electrophoretic mobility is unreliable, and the relation between the 33 kDa protein and the 34-32 kDa polypeptide deficient in certain photosystem II mutants has not been established. It was recently suggested (79) that this 34-32 kDa polypeptide is an intrinsic

polypeptide, and thus clearly distinct from the 33 kDa protein. Since an intrinsic polypeptide of around 34 kDa has been observed in photosystem II preparations from maize (79) and spinach (140), it is possible that both the extrinsic 33 kDa protein and the intrinsic 34 kDa polypeptides are involved in the oxygen evolving complex. Since the 33 kDa protein has been immunologically identified in both maize (Westhoff, P., personal communication) and *Chlamydomonas* (Andersson, B., personal communication), immunological studies on the maize and algal mutants should prove very informative.

Since only small reconstitutions with the 33 kDa protein have been obtained, functional studies are greatly hampered. Simple readdition of manganese and the 33, 23 and 16 kDa proteins to Tris-washed inside-out thylakoids did not restore oxygen evolution (Jansson, C., Åkerlund, H.-E. and Andersson, B. unpublished observation), although both proteins readily rebound to the depleted membranes (VII). A minor restoration of oxygen evolution was obtained in photosystem II detergent preparations by readdition of the released 33 kDa protein after treatments with high concentrations of urea (139), or CaCl_2 (114). These treatments released the 33, 23 and 16 kDa proteins but not the manganese. One model which is consistent with these results is that the 33 kDa protein participates in the coordination of manganese, together with some intrinsic polypeptide. This would also explain the ambiguity in the correlation between the 33 kDa protein and manganese. Such an arrangement, where two polypeptides cooperate in the formation of a binding domain, has been considered for the hemes in cytochrome oxidase (142). It should be remembered, that there may be more than one pool of manganese in the oxygen evolving complex. EPR measurements by Yocum et al. (36) indicated two pools of manganese, each containing two manganese. This is in line with a recent model proposed by Packham and Barber (143). They suggested that two manganese are required structurally to connect the oxygen evolving complex to the photosystem II reaction center

core. Thus, not all manganese in the oxygen evolving complex need to be catalytically involved in the oxidation of water. If such a heterogeneity exists, different polypeptides may be involved in the binding of the two pools of manganese.

5.6.3. The 16 kDa protein

In the reconstitution experiments in paper III no requirement for the 16 kDa protein could be demonstrated. Since the maximal reconstitution with the 23 kDa protein was around 50% (III,IV), it could be argued that some component other than the 23 kDa protein is additionally required for full restoration of oxygen evolution. The obvious candidate then would be the 16 kDa protein, but so far this has not been experimentally supported. The possibility that the 16 kDa protein was deactivated during the isoaltion procedure is less likely. Reconstitution with the 23 + 16 kDa proteins contained in the supernatant after the NaCl treatment, yielded the same maximal reconstitution as did the 23 kDa protein alone. The rebinding experiments in paper III and VII show that the 16 kDa protein could rebind to the membrane. Later studies by Ljungberg et al. (116), Miyao and Murata (136) and Andersson et al. (121) are all in agreement with the finding in paper III, that the 16 kDa protein does not contribute to any stimulation in oxygen evolution. In sharp contrast, Fukutaka et al. (120) reported restoration of oxygen evolution that required the presence of both the 23 and 16 kDa proteins. They extracted thylakoid membranes with cholate and high concentrations of NaCl to release the 33, 23 and 16 kDa proteins. The extracted membranes were then incubated with the purified 23 and 16 kDa proteins together with glycerol and cholate and spun down. This procedure restored half of the lost activity. The 23 or the 16 kDa protein alone had no effect.

The results of Fukutaka et al. (120) clearly show the requirement for the 16 kDa protein in oxygen evolution.

Noticeably, their activity measurements were carried out in the absence of added Cl^- . Thus, it is possible that the 16 kDa protein is required only at very low Cl^- concentrations. Another possible function of the 16 kDa protein, mentioned in the previous section, is as a Ca^{2+} -binding protein. The presence of calmodulin or a calmodulin-like protein in the oxygen evolving complex has been suggested (Section 3.3.). Calmodulin has also been isolated from spinach leaves (145,146) and green algae (146), and a 16-17 kDa protein isolated from chloroplasts showed calmodulin-like properties (Section 3.3.). However, calmodulin is a very conservative protein (147), and a comparison of the amino acid composition between the acidic calmodulin (146,147) and the basic 16 kDa protein (V), clearly reveals that the 16 kDa protein is not calmodulin. This does not exclude that the 16 kDa protein is a Ca^{2+} -binding protein, or that it is indirectly involved in the binding of Ca^{2+} in the oxygen evolving complex.

It is very interesting to note, that among the various photosystem II mutants of *Oenothera*, one mutant was found which specifically lacked the 16 kDa protein (Westhoff, P., personal communication). Other photosystem II polypeptides, including the 33 and 23 kDa proteins, cytochrome b_{559} and both the reaction center polypeptides, were present at wild type levels. Functional analyses of this mutant should yield crucial information about the role of the 16 kDa protein in the oxygen evolving process.

5.7. Topography

In paper III it was demonstrated that the 23 and 16 kDa proteins could rebind to the NaCl-treated inside-out thylakoids, when added under low ionic conditions. In paper VII, the sequential rebinding of the 33, 23 and 16 kDa proteins to Tris- and NaCl-treated inside-out thylakoids was systematically studied, using rocket immunoelectrophoresis. The conclusions from this study, and from previous

results, are embodied in the model of Fig. 7. The 33 kDa protein could rebind quite independently of the other two proteins. This is consistent with the release pattern after NaCl treatment (III) and shows that the 33 kDa protein is bound to some intrinsic polypeptide or directly to the lipid bilayer. The rebinding of the 23 kDa protein was strongly governed by the 33 kDa protein, suggesting that the 23 kDa protein is bound to the 33 kDa protein. Since, under certain conditions, the 23 kDa protein could be retained on the membrane also after release of the 33 kDa protein (39, Andersson, B., Klein-Hitpass, L., Jansson, C. and Berzborn, R., unpublished observation) it is assumed that the 23 kDa protein has binding sites also on components other than the 33 kDa protein. Although the isolated 33 kDa protein could rebind to almost control level, it was less effective in facilitating binding of the 23 kDa protein than was the unreleased 33 kDa protein. This indicates that the proper rebinding of the 33 kDa protein requires some conformational changes, or that the protein was partially denatured during isolation. The rebinding pattern for the 16 kDa protein shows a poor rebinding in the absence of the 23 kDa protein and a marked enhancement after addition of the 23 kDa protein. This suggests that the 16 kDa protein is bound to the 23 kDa protein. The high polarity index for the 33, 23 and 16 kDa proteins (IV,V) and their solubility without detergents (II,III) suggest that electrostatic interactions predominate in the binding to the membrane. This is especially true for the 23 and 16 kDa proteins, considering their ready release upon NaCl treatment (III,VII). For the 33 kDa protein, the release at elevated temperatures (140), and at alkaline pH (II,39,114,133,138) indicate contribution from hydrogen bonding. The release upon treatment with chaotropic agents (39) also suggests hydrophobic interactions. Although the 33 kDa protein is more firmly attached to the thylakoid membrane than the 23 and 16 kDa proteins, it is still highly exposed at the inner surface. This follows from its sensitivity to tryptic digestion (II) and reactivity with antibodies (VII).

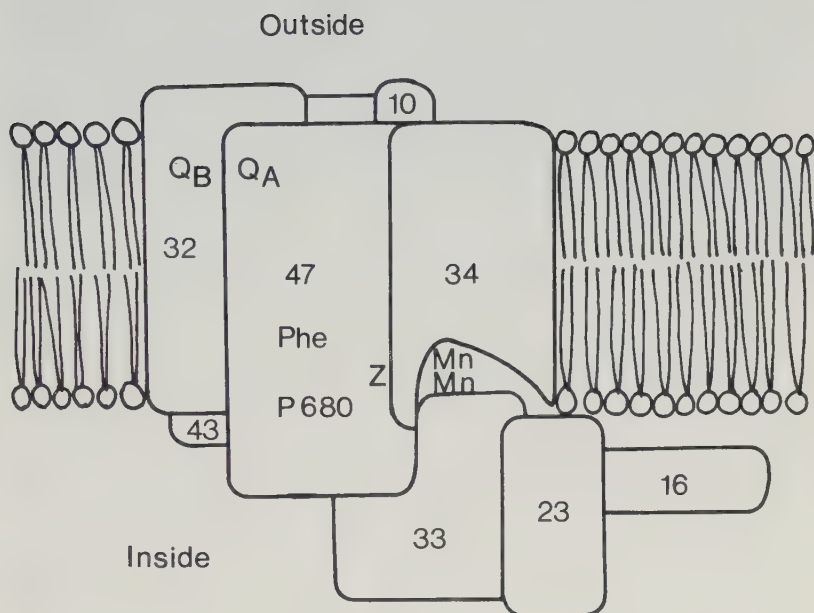


Fig. 7. A tentative model of the polypeptide composition on the donor side of photosystem II. The 47, 43, 34, 32 and 10 kDa polypeptides belong to the intrinsic photosystem II reaction center core complex, while the 33, 23 and 16 kDa proteins are extrinsically bound at the inner thylakoid surface.

The model in Fig 7 also shows the intrinsic photosystem II reaction center core complex. The reaction center preparation of Satoh and Butler (148) has been shown to contain five polypeptides (149); The two reaction center polypeptides of 50-43 kDa, the atrazine-binding 32 kDa polypeptide, a 34 kDa polypeptide and a 10 kDa polypeptide belonging to cytochrome b_{559} . The 49 kDa polypeptide probably

binds both P680 and the immediate acceptor pheophytin (150) and possibly also the primary quinone acceptor Q_A and the species Z. The 43 kDa polypeptide has been suggested to serve in the binding of antenna chlorophyll a (150). The atrazine-binding 32 kDa polypeptide has been identified (149) as the B-protein, harbouring the secondary quinone acceptor Q_B . The 34 kDa polypeptide may be identical to the intrinsic polypeptide suggested to be involved in the binding of manganese (Section 5.6.2.), but has also been considered as the Z-binding protein (149).

The location and number of manganese in the oxygen evolving complex is a matter of speculation and the present model is intended to leave much of these questions open. The suggested arrangement, with the participation of both the 34 and 33 kDa polypeptides in the coordination of manganese, is one likely alternative which tries to accommodate most of the recent findings. It is noteworthy, that if any of the intrinsic core polypeptides is involved in the coordination of the catalytic manganese, this would imply that there is no discrete oxygen evolving complex within the donor side of photosystem II, but rather, the entire donor side is the oxygen evolving complex. However, it should be emphasized that the model in Fig. 7 is simplified, and additional polypeptides in the oxygen evolving complex, apart from the 33, 23 and 16 kDa proteins, have recently been implicated from immunoprecipitation studies by Ljungberg et al. (124).

The concept of the water-oxidizing site residing in a sequestered domain in the thylakoid membrane, originates from the studies by Dilley and coworkers (151,152) and has during the past several years been independently supported in a number of studies (153-158). The main argument for this concept is that the protons derived from water oxidation do not readily equilibrate with the inner bulk phase, but seem to be separated from the lumen by a proton-impermeable barrier (151-156). This inference has been supported by studies on release of Cl^- (153,155,157) and manganese (158). Although from a quite different stand-

point, also the alternative model to the Z scheme, proposed by Arnon et al. (159), suggests a sequestered location of the water-oxidizing site (159,160). The original interpretation by Dilley and coworkers (151) of the water-oxidizing site in an intramembraneous domain, is difficult to reconcile with the present knowledge of the oxygen evolving complex (I-III, VI,VII). Recently however, models have been proposed where one or some of the extrinsic 33, 23 and 16 kDa proteins participate in the formation of a sequestered domain around the water-oxidizing site (38, 161). In the present model, the structural constraints of this domain is a crevice, formed mainly by the 34, and 33 kDa polypeptides. However, the 23 and 16 kDa proteins might also be involved.

5.8. Biogenesis

Using in vitro translation and immunoprecipitation, we could demonstrate that the 33, 23 and 16 kDa proteins are all encoded by nuclear DNA and synthesized on cytoplasmic ribosomes as precursors, 6-10 kDa larger than the mature proteins (Fig. 8). We also showed that the in vitro products could be post-translationally imported into isolated intact chloroplasts, and most likely reach their final destination at the inner thylakoid surface. During or after this translocation the precursor proteins were processed to their mature forms. Hence, the in vitro products contained all information for transport across three membranes, for processing and probably also for the assembly into functional units. An interesting question, which this study was not designed to answer, is if the processing of the precursor 33, 23 16 kDa proteins occurs in two steps, the first at the translocation across the envelope membranes and the second at the translocation across the thylakoid membrane. The large size of the transit sequences suggest such a two-step processing. Since both the reaction center polypeptides (162), the B-protein (163-165) and cytochrome b_{559} (166) are encoded by the chloro-

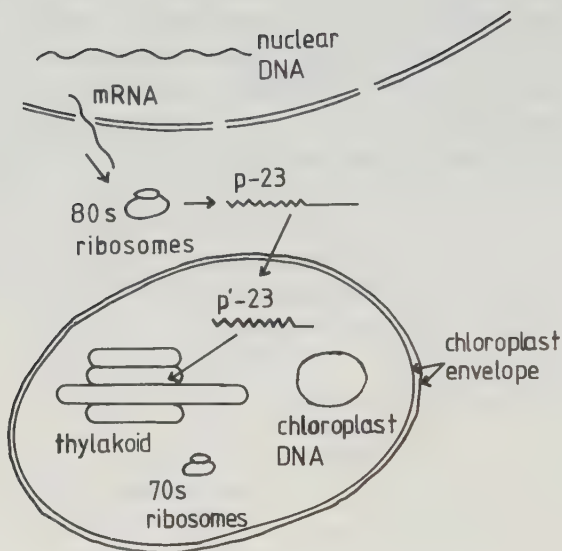


Fig. 8. Biosynthesis and transport of the nuclear-encoded 33, 23 and 16 kDa proteins. P denotes the precursor protein with a 6-10 kDa transit sequence. P' is a hypothetical intermediary stage in the processing of the precursor. An alternative model is that the translocation across the envelope and thylakoid membranes occurs in one step at a membrane junction. After translocation across the thylakoid membrane, the 33, 23 and 16 kDa proteins are assembled with chloroplast-encoded polypeptides of photosystem II.

plast DNA, our results imply that, like for other multi-component complexes in the thylakoid membrane, the biogenesis of photosystem II is the result of a fascinating and intricate interplay between the nuclear and chloroplast genomes.

6. EPILOGUE

When examining the activities of the oxygen evolving complex, and of its individual constituents, it should be borne in mind that certain mechanisms may not be operating under in vitro conditions. One such aspect is the response of the oxygen evolving complex to fluctuations in the lumenal pH. In the intact chloroplast the internal pH in the thylakoid lumen reaches around 4.5 in light (167). In addition, the pH gradient of 3-4 units (168) generated over the thylakoid membrane, is compensated by an influx of Cl^- and an efflux of Mg^{2+} (169). It is reasonable to assume that the acidic conditions in the thylakoid lumen during photosynthesis, as well as the ion fluxes, essentially affect the oxygen evolving complex and allert regulatory action. A second, related aspect, indicated in Section 5.7., is that the oxygen evolving complex may participate in a processing of protons derived from water oxidation. A third aspect concerns the feedback systems that would be expected to operate in the stroma. Responding to levels of CO_2 , NADPH, and ATP, the electron flow between the two photosystems may disconnect and switch over to a cyclic mode around photosystem I and probably also around photosystem II. A cyclic electron flow around photosystem II has been repeatedly suggested (70,74-77) and the feedback regulation of such a cycle via cytochrome b_{559} has been inferred from studies on green algae (75). A fourth aspect is the light-induced phosphorylation of thylakiod polypeptides (170,171). Phosphorylation of polypeptides in the light-harvesting complex of photosystem II seems to control the distribution of absorbed excitation energy between the two photosystems (169-171). However, also other photosystem II polypeptides are phosphorylated and very little is known about the function of this phosphorylation, although some progress has been made (76,173). A fifth and final aspect is the obvious reflection, that every activity which is dependent on a closed, right-side out thylakoid structure will escape detection in inside-out thylakoids and photosystem II detergent pre-

parations. The five examples above point to the possibility that there may be regulatory components in the oxygen evolving complex that are normally silent, or only partially required, in sub-chloroplast preparations, and even more so in inside-out thylakoids and other photosystem II preparations. Although this conclusion appears quite trivial, it should prove fruitful to consider in future experimentation.

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REFERENCES

1. Clayton, R.K. (1980) in *Photosynthesis II: Physical mechanisms and chemical patterns*, pp 33, Cambridge University press
2. Margulis, L. (1981) in *Symbiosis in Cell Evolution*, pp 10, W.H Freeman and Company
3. Joliot, P. (1960) Thèse de Doctorat es Sci. Phys., Paris, France
4. Joliot, P. (1968) *Photochem. Photobiol.* 8, 451
5. Joliot, P. and Joliot, A. (1968) *Biochim. Biophys. Acta* 153, 625-652
6. Joliot, P., Barbieri, G. and Chabaud, R. (1969) *Photochem. Photobiol.* 10, 309-329
7. Kok, B., Forbush, B. and McGloin, M. (1970) *Photochem. Photobiol.* 11, 457-475
8. Brudvig, G.W., Casey, J.L. and Sauer, K. (1983) *Biochim. Biophys. Acta* 723, 366-371
9. Ghanotakis, D.F., O'Malley, P.J., Babcock, G.T. and Yokum, C.F. (1983) in *The Oxygen Evolving System of Photosynthesis* (Y. Inoue et al., eds) pp 87-98, Academic Press, Tokyo
10. Babcock, G.T. and Sauer, K. (1975) *Biochim. Biophys. Acta* 376, 329-244
11. Blankenship, R.E., Babcock, G.T., Warden, J.T. and Sauer, K. (1975) *FEBS Lett.* 51, 287-293
12. Warden, J.T., Blankenship, R.E. and Sauer, K. (1976) *Biochim. Biophys. Acta* 423, 462-478
13. Babcock, G.T., Blankenship, R.E. and Sauer, K. (1976) *FEBS Lett.* 61, 286-289
14. Dekker, J.P. van Gorkom, H.J. Brok, M. and Ouwehand, L. (1984) *Biochim. Biophys. Acta*, in press
15. Bouges-Bocquet, B. (1980) *Biochim. Biophys. Acta* 594, 85-107
16. Joliot, P. and Kok, B. (1975) in *Bioenergetics of Photosynthesis* (Govindjee, ed.) pp 387-412, Academic Press, New York
17. Sauer, K. (1980) *Accounts of Chemical Research* 13, 249-256

18. Brettel, K., Schlodder, E. and Witt, H.T. (1984) in Proceedings of the 6th International Congress on Photosynthesis (Sybesma, C., ed.) Martinus Nijhoff, the Hague, in press
19. Förster, V., Hong, Y.-Q. and Junge, W. (1981) Biochim. Biophys. Acta 638, 141-152
20. Wille, B. and Lavergne, J. (1982) Photobiochem. Photobiophys. 4, 131-144
21. Fowler, C.F. (1977) Biochim. Biophys. Acta 462, 414-421
22. Saphon, S. and Crofts, A. R. (1977) Z. Naturforsch. 32c, 617-626
23. Velthuys, B.R. (1980) FEBS Lett. 115, 167-170
24. Wydrzynski, T. and Sauer, K. (1980) Biochim. Biophys. Acta 589, 56-70
25. Renger, G. and Echert, H.J. (1980) Bioelectrochem. Bioenerg. 7, 101-124
26. Dismukes, G.C., Ferris, K. and Watnick, P. (1982) Photobiochem. Photobiophys. 3, 243-256
27. Renger, G., Eckert, H.-J. and Weiss, W. (1983) in The Oxygen Evolving System of Photosynthesis (Y. Inoue et al., eds) pp 73-82, Academic Press, Tokyo
28. Kusunoki, M. (1983) Ibid pp 165-173
29. Skoog, D.A. and West, D.M. (1976) in Fundamentals of analytical chemistry, p 780, Holt, Reinhart and Winston
30. Wydrzynski, T., Zumbulyadis, W., Schmidt, P.G., Gutowsky, H.S. and Govindjee (1976) Proc. Natl. Acad. Sci. USA 73, 1196-1198
31. Dismukes, G.C. and Siderer, Y. (1980) FEBS Lett. 121, 78-80
32. Dismukes, G.C. and Siderer, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 274-278
33. Hansson, Ö. and Andréasson, L.-E. (1982) Biochim. Biophys. Acta 679, 261-268
34. Dismukes, G.C., Abramowicz, D.A., Ferris, K.F., Mathur, P., Siderer, Y., Upadrashta, B. and Watnick, P. (1983) in The Oxygen Evolving System of Photosynthesis (Y. Inoue et al., eds) pp 145-158 Academic Press, Tokyo

35. Cheniaie, G. M. and Martin, I.F. (1970) *Biochim. Biophys. Acta* 197,219-239
36. Yocum, C.F., Yerkes, C.T., Blankenship, R.E., Sharp, R.R. and Babcock, G.T. (1981) *Proc. Natl. Acad. Sci. USA* 78,7507-7511
37. Ames, J. (1983) *Biochim. Biophys. Acta* 726, 1-12
38. Cammarata, K., Tamura, N., Sayre, R and Cheniaie, G. (1984) in *Proceedings of the 6th International Congress on Photosynthesis* (Sybesma, C., ed.) Martinus Nijhoff, the Hague, in press
39. Murata, N., Miyao, M. and Kuwabara, T. (1983) in *The Oxygen Evolving System of Photosynthesis* (Y. Inoue et al., eds) pp 213-222 Academic Press, Tokyo
40. Yamamoto, Y. and Nishimura, T. (1983) *Biochim. Biophys. Acta* 724,294-297
41. Yamamoto, Y. and Nishimura, T. (1983) in *The Oxygen Evolving System of Photosynthesis* (Y. Inoue et al., eds) pp 229-238, Academic Press, Tokyo
42. Wargurg, O. and Luttgens, W. (1944) *Naturwissenschaften* 32, 301
43. Izawa, S., Heath, R.C. and Hind, G. (1969) *Biochim. Biophys. Acta* 180, 388-398
44. Theg, S.M. and Homann, P.H. (1982) *Biochim. Biophys. Acta* 679, 221-234
45. Critchley, C., Baianu, I.G., Govindjee and Gutowsky, H.S. (1982) *Biochim. Biophys. Acta* 682, 436-445
46. Muallem, A., Farineau, J., Laine-Boszormenyi, M. and Izawa, S. (1981) in *Proceedings of the 5th International Congress on Photosynthesis* (Akoyunoglou, G., ed.) vol. 2, pp 435-443 Balaban International Science Services, Philadelphia, PA
47. Muallem, A. and Laine-Boszormenyi, M. (1981) *Photo-biochem. Photobiophys.* 2, 337-343
48. Izawa, S., Muallem, A. and Ramaswamy, N.K. (1983) in *The Oxygen Evolving System of Photosynthesis* (Y. Inoue et al., eds) pp 291-302, Academic Press, Tokyo
49. Govindjee, Bainu, I.C., Chritchley, C. and Gutowsky, H.S. (1983) *Ibid*, pp 303-315
50. Sandusky, P. and Yokum, C.F. (1983) *FEBS Lett.* 162, 339-343

51. Critchley, C. (1983) *Biochim. Biophys. Acta* 724, 1-5
52. Ramaswamy, N.K. and Izawa, S. (1983) *Plant Physiol.* 72, (suppl.) 56
53. Ono, T.-A. and Inoue, Y. (1983) *Biochim. Biophys. Acta* 723, 191-201
54. Ono, T.-A. and Inoue, Y. (1983) in *The Oxygen Evolving System of Photosynthesis* (Y. Inoue et al., eds) pp 337-344, Academic Press, Tokyo
55. Yamashita, T. and Tomita, G. (1974) *Plant Cell Physiol.* 15, 69-82
56. Yamashita, T. and Tomita, G. (1976) *Plant and Cell Physiol.* 17, 571-582
57. Yamashita, T. and Ashizawa, A. (1983) in *The Oxygen Evolving System of Photosynthesis* (Y. Inoue et al., eds) pp 327-336, Academic Press, Tokyo
58. England, R.R. and Evans, E.H. (1983) *Biochem. J.* 210, 473-476
59. Yerkes, C.T. and Babcock, G.T. (1981) *Biochim. Biophys. Acta* 634, 19-39
60. Nakatani, H.Y. (1984) *Biochim. Biophys. Acta*, in press
61. Barr, R., Troxel, K.S. and Crane, F.L. (1982) *Biochem. Biophys. Res. Commun.* 104, 1182-1188
62. Barr, R. and Crane, F.L. in *Proceedings of the 6th International Congress on Photosynthesis* (Sybesma, C., ed.) Martinus Nijhoff, the Hague, in press
63. Cramer, W.A. and Horton, P. (1975) *Photochem. Photobiol.* 22, 304-308
64. Cramer, W.A. and Whitmarsh, J. (1977) *Annu. Rev. Plant Physiol.* 28, 133-172
65. Bendall, D.S. (1982) *Biochim. Biophys. Acta* 683, 119-151
66. Butler, W.L. (1978) *FEBS Lett.* 95, 19-25
67. Metz, J.G., Wong, J. and Bishop, N.I. (1980) *FEBS Lett.* 114, 61-66
68. Metz, J. and Bishop, N.I. (1980) *Biochem. Biophys. Res. Commun.* 94, 560-566
69. Bishop, N.I. (1983) in *The Oxygen Evolving System of Photosynthesis* (Y. Inoue et al., eds) pp 177-187 Academic Press, Tokyo

70. Heber, U., Kirk, M.R. and Boardman, W.K. (1979) *Biochim. Biophys. Acta* 546, 297-306
71. Cox, R.P. and Bendal, D.S. (1972) *Biochim. Biophys. Acta* 283, 124-135
72. Gerhardt, B. (1964). *Planta* 61, 101-129
73. Butler, W.L., Matsuda, H. (1983) in *The Oxygen Evolving System of Photosynthesis* (Y. Inoue et al., eds) pp 113-122 Academic Press, Tokyo
74. Henningsen, K.W. and Boardman, N.K. (1973) *Plant Physiol.* 51, 1117-1126
75. Mende, D. and Wiessner, W. (1983) *Photobiochem. Photobiophys.* 6, 1-7
76. Jursinic, P.A. and Kyle, D.J. (1983) *Biochim. Biophys. Acta* 723, 37-44
77. Horton, P. and Lee, P. (1983) *FEBS Lett.* 162, 81-84
78. Ford, R.C. and Evans, M.C.W. (1983) *FEBS Lett.* 160, 159-164
79. Bricker, T. M., Metz, J.G., Miles, D. and Sherman, L.A. (1983) *Biochim. Biophys. Acta* 724, 447-455
80. Sadusky, P.O., Selvius De Roo, C.L., Hicks, D. B., Yokum, C.F., Ghanotakis, D.F. and Babcock, G.T. (1983) in *The Oxygen Evolving System of Photosynthesis* (Y. Inoue et al., eds) pp 189-199 Academic Press, Tokyo
81. Erixon, K. and Butler, W.L. (1971) *Biochim. Biophys. Acta* 234, 381-389
82. Widger, W.R., Cramer, W.A., Hermodson, M., Gullifor, M., Meyer, D., Farchaus, J. and Liedtke, B. (1983) in *The Oxygen Evolving System of Photosynthesis* (Y. Inoue et al., eds) pp 123-134 Academic Press, Tokyo
83. Spector, M. and Winget, G.D. (1980) *Proc. Natl. Acad. Sci. USA* 77, 957-959
84. Winget, G.D. and Spector, M.S. (1981) in *Proceedings of the 5th International Congress on Photosynthesis* (Akoyunoglou, G., ed.) vol. 2, pp 453-464 Balaban International Science Services, Philadelphia, PA
85. Nakatani, H., Barber, J. and Evans, J. (1981) *Ibid* pp 487-494
86. Nakatani, H., and Barber, J. (1981) *Photobiochem. Photobiophys.* 2, 69-78
87. Okada, S. and Asada, K. (1983) *Plant Cell Physiol.* 24, 163-172

88. Asada, K. and Okada, S. (1983) in *The Oxygen Evolving System of Photosynthesis* (Y. Inoue et al., eds) pp. 245-255 Academic Press, Tokyo
89. Lagoutte, B. and Duranton, J. (1975) *FEBS Lett.* 51, 21-24
90. Foyer, C.H. and Hall, D.O. (1979) *FEBS Lett.* 101, 324-338
91. Fredricks, W. and Jagendorf, A. (1964) *Arch. Biochem. Biophys.* 104, 39-49
92. Tel-Or, E. and Avron, M. (1975) in *Proceedings of the 3rd International Congress on Photosynthesis* (Avron, M., ed.) pp 569-578, Elsevier, Amsterdam
93. Huzisige, H., Isomoto, M. and Inoue, H. (1968) in *Comparative Biochemistry and Biophysics of Photosynthesis* (Shibata, K., ed.) pp 170-178, University of Tokyo Press, Tokyo
94. Andersson, B., Åkerlund, H.-E. and Albertsson, P.-Å. (1977) *FEBS Lett.* 77, 141-145
95. Andersson, B. and Åkerlund, H.-E. (1978) *Biochim. Biophys. Acta* 503, 462-472
96. Yamamoto, Y., Doi, M., Tamura, W. and Nishimura, M. (1981) *FEBS Lett.* 133, 265-268
97. Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) *FEBS Lett.* 134, 231-234
98. Kuwabara, T. and Murata, N. (1982) *Plant Cell Physiol.* 23, 532-539
99. Andersson, B., Simpson, D.J. and Hoyer-Hansen, G. (1978) *Carlsberg Res. Commun.* 43, 77-89
100. Andersson, B., Sundby, C. and Albertsson, P.-Å. (1980) *Biochim. Biophys. Acta* 599, 391-402
101. Albertsson, P.-Å., Andersson, B., Larsson, C. and Åkerlund, H.-E. (1982) in *Methods of Biochemical Analysis*, vol 28, pp 115-150
102. Dunahay, T.G., Staehlin, L.A. Seibert, M., Ogilvie, P.D. and Berg, S. P. (1984) *Biochim. Biophys. Acta* 764, 179-193
103. Lindberg-Møller, B. and Høj, P.B. (1983) *Carlsberg Res. Commun.* 48, 161-185
104. Gounaris, K., Whitford, D. and Barber, J. (1984) *FEBS Lett.* in press
105. Ausländer, W. and Junge, W. (1975) *FEBS Lett.* 59, 310-315

106. Blankenship, R.E. and Sauer, K. (1974) *Biochim. Biophys. Acta* 357, 252-266
107. Selman, B.R. and Bannister, T.T. (1971) *Biochim. Biophys. Acta* 253, 428-436
108. Selman, B.R., Bannister, T.T. and Dilley, R.A. (1973) *Biochim. Biophys. Acta* 292, 566-581
109. Giaquinta, R.T., Dilley, R.A. and Anderson, B. J. (1973) *Biochem. Biophys. Res. Commun.* 52, 1410-1417
110. Yerkes, C.T. and Babcock, G.T. (1980) *Biochim. Biophys. Acta* 590, 360-372
111. Kuwabara, T. and Murata, N. (1979) *Biochim. Biophys. Acta* 581, 228-236
112. Cheniae, G.M. and Martin, I.F. (1978) *Biochim. Biophys. Acta* 502, 321-344
113. Frash, W.D. and Cheniae, G.M. (1980) *Plant Physiol.* 65, 735-745
114. Ono, T.-A. and Inoue, Y. (1984) *FEBS Lett.* 166, 381-384
115. Åkerlund, H.-E. (1981) in *Proceedings of the 5th International Congress on Photosynthesis* (Akoyunoglou, G., ed.) vol. 2, pp 465-472 Balaban International Science Services, Philadelphia, PA
116. Ljungberg, U., Jansson, C., Andersson, B. and Åkerlund, H.E. (1983) *Biochem. Biophys. Res. Commun.* 113, 738-744
117. Åkerlund, H.-E., Brettel, K. and Witt, H.T. (1984) *Biochim. Biophys. Acta*, in press
118. Yamamoto, Y., Shimada, S. and Nishimura, M. (1983) *FEBS Lett.* 151, 49-53
119. Kuwabara, T. and Murata, N. (1983) in *Proceedings of the 6th International Congress on Photosynthesis* (Sybesma, C., ed.) Martinus Nijhoff, the Hague, in press
120. Fukutaka, F., Imaoka, A., Akabori, K. and Toyoshima, Y. (1983) *FEBS Lett.* 158, 217-221
121. Andersson, B., Critchley, C., Ryrie, I.J., Jansson, C., Larsson, C. and Andersson, J.M. (1984) *FEBS Lett.* 168, 113-117
122. Westhoff, P., Jansson, C., Klein-Hitpass, L., Berzborn, R., Larsson, C. and Bartlett, S.G. (1984) *Plant Molecular Biology*, in press

123. Larsson, C., Jansson, C., Ljungberg, U., Åkerlund, H.-E. and Andersson, B. (1984) in Proceedings of the 6th International Congress on Photosynthesis (Sybesma, C., ed.) Martinus Nijhoff, the Hague, in press
124. Ljungberg, U., Jansson, C., Larsson, C., Åkerlund, H.-E. and Andersson, B. (1984) *Ibid*
125. Kyle, D.J., Ohad, I., Guy, R. and Arntzen, C.J. (1983) in The Oxygen Evolving System of Photosynthesis (Y. Inoue et al., eds) pp 401-416
126. Stewart, A.C. and Bendal, D.S. (1981) *Biochem. J.* 194, 877-887
127. Anderson, J.M. and Melis, A. (1983) *Proc. Natl. Acad. Sci. USA* 88, 745-749
128. Anderson, B. and Haehnel, W. (1983) *FEBS Lett.* 146, 13-17
129. Andersson, B. (1984) in Proceedings of the 6th International Congress on Photosynthesis (Sybesma, C., ed.) Martinus Nijhoff, the Hague, in press
130. Babcock, G.T. and Sauer, K. (1975) *Biochim. Biophys. Acta* 396, 48-62
131. Wensink, J. and Van Gorkom, H.J. (1984) in Proceedings of the 6th International Congress on Photosynthesis (Sybesma, C., ed.) Martinus Nijhoff, the Hague, in press
132. Ghanotakis, D.F., Babcock, G.T. and Yocum, C. F. (1984) *FEBS Lett.* 167, 127-130
133. Ono, T.-A. and Inoue, Y. (1983) *FEBS Lett.* 164, 255-260
134. Ghanotakis, D.F., Topper, J. W., Babcock, G.T. and Yocum, C.F. (1984) *FEBS Lett.* in press
135. Miyao, M. and Murata, N. (1984) *FEBS Lett.* in press
136. Miyao, M. and Murata, N. (1983) *Biochim. Biophys. Acta* 725, 87-93
137. Williams, R.J.P. (1982) *FEBS Lett.* 140, 3-10
138. Kuwabara, T. and Murata, N. (1983) *Plant Cell Physiol.* 24, 741-747
139. Miyao, M. and Murata, N. (1983) *FEBS Lett.* 164, 375-378
140. Franzen, L.-G. and Andréasson, L.-E. (1984) *Biochim. Biophys. Acta*, in press

141. Metz, J.G. and Miles, D. (1980) *Biochim. Biophys. Acta* 681, 95-102
142. Wikström, M., Krab, K. and Saraste, M. (1981) in *Cytochrome Oxidase; A synthesis*, p 37, Academic Press, London
143. Packam, N.K. and Barber, J. (1984) *Biochim. Biophys. Acta* 764, 17-23
144. Watterson, D.M., Burgess, W.H., Lukas, T.J., Ivererson, D., Marshak, D.R., Schleicher, M., Erickson, B.W., Fok, K.-F. and Van Eldick, L.J. (1983) *Advan. Cycl. Nucl. Res.* 16, 205-226
145. Burgess, W.H., Schleicher, M., Van Eldick, L.J. and Watterson, D.M. (1983) in *Calcium and Cell Function* (Cheung, W.-Y., ed.) vol. 4, pp 269-261, Academic Press, New York
146. Schleicher, M., Lukas, T.J. and Watterson, D.M. (1984) *Arch. Biochem. Biophys.* 229, 33-42
147. Klee, C.B., Crouch, T.H. and Richman, P.G. (1980) *Ann. Rev. Biochem.* 49, 489-515
148. Satoh, K. and Butler, W.C. (1978) *Plant Physiol.* 61, 373-379
149. Satoh, K., Nakatani, H.Y., Steinback, K.E., Watson, J. and Arntzen, C.J. (1983) *Biochim. Biophys. Acta* 724, 142-150
150. Nakatani, H.Y. (1984) in *Proceedings of the 6th International Congress on Photosynthesis* (Sybesma, C., ed.) Martinus Nijhoff, the Hague, in press
151. Prochaska, L.J. and Dilley, R.A. (1978) *Biochem. Biophys. Res. Commun.* 83, 664-672
152. Baker, G.M., Bhatnagar, D. and Dilley, R.A. (1981) *Biochemistry* 20, 2307-2315
153. Theg, S.M. and Homann, P.H. (1982) *Biochim. Biophys. Acta* 679, 221-234
154. Theg, S.M., Johnson, J.D. and Homann, P.H. (1983) *FEBS Lett* 145, 25-29
155. Johnson, J.D., Pfister, V.R. and Homann, P.H. (1983) *Biochim. Biophys. Acta* 723, 256-265
156. Theg, S.M. and Junge, W. (1983) *Biochim. Biophys. Acta* 723, 294-307
157. Richter, M., Johnson, J.D. and Homann, P.H. in *Proceedings of the 6th International Congress on Photosynthesis* (Sybesma, C., ed.) Martinus Nijhoff, the Hague, in press

158. Miller, M. and Cox, R.P. (1984) *Biochem. Biophys.-Res. Commun.* 119, 168-173
159. Arnon, D.I., Tsujimoto, H.Y. and Tang, G.M.-S. (1981) *Proc. Natl. Acad. Sci. USA* 78, 2942-2946
160. Albertsson, P.-Å., Hsu, B.-O., Tang, G.M.-S. and Arnon, D.I. (1983) *Proc. Natl. Acad. Sci. USA* 80, 3971-3975
161. Barber, J. (1984) *Trends in Biochem. Sci.* 9, 79-80
162. Westhoff, P., Alt, J. and Herrmann, R.G. (1983) *The EMBO Journal* 2, 2229-2237
163. Bedbrock, J.R., Link, G., Coen, D.M., Bogorad, L. and Rich, A. (1978) *Proc. Natl. Acad. Sci. USA* 75, 3060-3064
164. Driesel, A.J., Speirs, J. and Bohnert, H.J. (1980) *Biochim. Biophys. Acta* 610, 297-310
165. Zurawski, G., Bohnert, H.J. Whitfeld, P.R. and Bot-
tomley, W. (1983) *Proc. Natl. Acad. Sci. USA* 79,
7699-7703
166. Zielinski, R.E. and Price, C.A. (1980) *J. Cell Biol.*
85, 435-445
167. Schuldiner, S., Rottenberg, H. and Avron, M. (1972)
Eur. J. Biochem. 25, 64-70
168. Avron, M. (1981) in *The Biochemistry of Plants*
(Hatch, M.D. and Boardman, N.K., ed.) vol. 8, p
181, Academic Press, New York
169. Vambutas, V. and Schechter, S. (1983) *Arch. Bioc-
hem. Biophys.* 224, 442-448
170. Haworth, P., Kyle, D., Horton, P. and Arntzen, C.J.
(1982) *Photochem. Photobiol.* 36, 743-748
171. Bennet, J. (1983) *Biochem. J.* 212, 1-13
172. Bennet, J., Steinback, K.E. and Arntzen, C.J.
(1980)
173. Owens, G.C. and Ohad, I. (1983) *Biochim. Biophys.
Acta* 722, 234-241

TRYPSINATION OF INSIDE-OUT CHLOROPLAST THYLAKOID VESICLES FOR LOCALIZATION OF THE WATER-SPLITTING SITE

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1. Introduction

Many conflicting results have been reported concerning the location of the water splitting site in the chloroplast thylakoid membrane [1,2]. An internal location has been assumed mainly from the internal release of protons linked to oxygen production [3,4] and the selective release of manganese into the luminal space upon Tris treatment [5]. In contrast, an external exposure has been inferred from the inactivation of photosystem II electron transport by chemical probes considered to be unable to penetrate the membrane [6,7]. A location at the outer surface has also been concluded from the trypsin digestion of the chloroplast lamellae [8,9] while the effect of trypsin has been claimed to be due mainly to proteolysis of the reducing side of photosystem II [10,11].

These conflicting results may reflect the inherent limitations in modifying only the outer membrane surface for studies on the transverse organization of a membrane. Modification of a component on the outer surface may cause rearrangements in the membrane which leads to inactivation of internal catalytic sites. However, the absence of effects does not reveal whether a component is located internally or just shielded behind some bulky membrane constituents. A direct comparison between the effects of non-penetrating reagents on the outer and inner membrane surfaces is therefore desirable. Such

a comparison has been possible for the chloroplast thylakoid membrane for the first time by the recent isolation of inside-out vesicles by two-phase partition [12–15]. Their reversed sidedness has been demonstrated by the direction of the proton and electrical gradients [12–14] and by freeze-fracture electron microscopy [15].

Here the trypsin effect on photosystem II electron transport has been compared for closed thylakoid membranes of opposite sidedness. The results show that DCIP reduction using water as electron donor was sensitive to trypsin in both types of membranes. However, upon addition of diphenyl carbazide the activity was completely restored in the inside-out but not in the rightside-out fraction. This strongly favours an internal location of the water splitting site of the photosynthetic electron transport.

2. Materials and methods

2.1. Preparation of thylakoid membrane fractions

Stacked spinach chloroplast lamellae (class II) [16] were fragmented by a Yeda press disintegration procedure [13]. Inside-out thylakoid vesicles formed during this procedure were separated from rightside-out material by partition in an aqueous dextran–polyethylene glycol two-phase system [13]. Chloroplast fragments (5 ml at 800 µg chl/ml) were added to 20 g polymer mixture to yield the following composition: 5.7% (w/w) dextran 500, 5.7% (w/w) polyethylene glycol 4000, 10 mmol/kg sodium phosphate buffer (pH 7.4), 5 mmol/kg NaCl, 20 mmol/kg sucrose and chloroplast material corresponding to 4 mg chl. The concentrations are based on the final weight of the

Abbreviations: chl, chlorophyll; DCIP, 2,6-dichlorophenol indophenol; DPC, 1,5-diphenyl carbazide

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phase system. The phase system was carefully mixed and allowed to separate. The top phase (T1) and bottom phase (B1) were collected and repartitioned with pure bottom phase and top phase, respectively, yielding fractions T2 and B2. The B2 fraction was purified by a further partition step giving a B3 fraction. The material in this fraction was removed from the viscous bottom phase by a final partition step with a top phase containing the positively charged trimethylamino-polyethylene glycol [17]. The B3 fraction contains the inside-out thylakoid vesicles while the T2 fraction contains vesicles of normal sidedness.

Rightside-out fraction destacked chloroplasts were mainly used. They were prepared from stacked chloroplast lamellae [16] by incubation for ≥ 30 min in 50 mM tricine (pH 7.4), 100 mM sucrose. Destacked chloroplasts were preferred since the partitions might restrict the enzyme action.

2.2. Trypsin treatment

Prior to trypsination all samples were set at 35 μ g chl/ml using the top phase from a system containing 5.7% (w/w) dextran 500, 5.7% (w/w) polyethylene glycol 4000, 50 mmol/kg tricine (pH 7.4) and 100 mmol/kg sucrose. Trypsination was performed at room temperature with a trypsin (Sigma type XI): chlorophyll ratio of 0.025:2. After 2.5 min digestion under magnetic stirring, soybean trypsin inhibitor (Sigma type I-S) was added to yield an inhibitor to trypsin ratio of 100. The control samples of each fraction received a mixture of trypsin and inhibitor yielding the same final concentration as for the digested samples.

2.3. Electron transport measurement

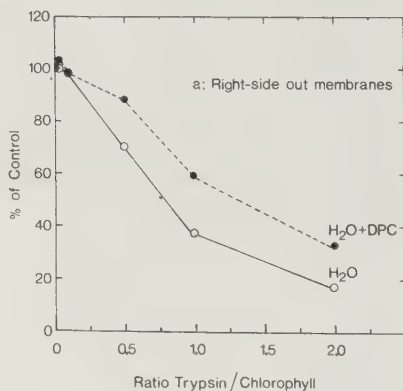
Photosystem II electron transport activities with DCIP as electron acceptor was followed at 550 nm using an Aminco DW-2 spectrophotometer operating in split beam mode at 20°C. The intensity of the actinic light ($\lambda > 610$ nm) was 160 W/m². As electron donors both water and diphenyl carbazide were used. The reaction mixtures are given in the legend to fig.1.

3. Results

The influence of trypsin on the rate of photo-

system II electron transport, measured as DCIP reduction, in the unbroken chloroplast lamellae and the inside-out fraction is shown in fig.1a and 1b, respectively. With water as electron donor a pronounced inhibition was observed both for unbroken chloroplast lamellae and inside-out thylakoid vesicles (B3). On addition of diphenyl carbazide the photosystem II activity of the inside-out vesicles was completely restored (fig.1b). In contrast, diphenyl carbazide had only a minor effect on the activity of the unbroken chloroplast lamellae (fig.1a). The same inhibitory pattern both in the presence and absence of diphenyl carbazide, as demonstrated for the unbroken chloroplast lamellae (fig.1a), was obtained for thylakoid vesicles of normal sidedness (T2, not shown). This result indicates that the differences in the effects of trypsin on rightside-out and inside-out material are due to differences in sidedness rather than size. The stimulation by diphenyl carbazide in the T2 fraction was slightly stronger than for chloroplast lamellae, which can be explained by the contamination of inside-out vesicles (10%) in the T2 fraction [15].

The higher control activity found for the inside-out material using diphenyl carbazide + water as electron donors, compared to the unbroken chloroplast lamellae, is in agreement with our previous finding that these vesicles are enriched in photosystem II [16]. Due to the Yeda press treatment



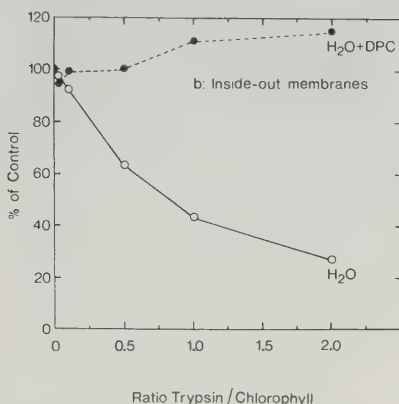


Fig.1. Effects of trypsin on DCIP reduction in chloroplast thylakoid membranes of opposite sidedness. The ΔA_{550} was measured in a suspension (pH 6.1) of the following composition: 40 mM sodium phosphate; 4.4 mM NaCl; 22 mM tricine; 44 mM sucrose; 36 μ M DCIP; chloroplast material corresponding to 6.7 μ g chl/ml and, where indicated, 1.1 mM diphenyl carbazide. The 100% rates for unbroken chloroplast lamellae were 156 μ mol DCIP reduced $\cdot$ mg chl⁻¹ $\cdot$ h⁻¹ both in the absence and presence of diphenyl carbazide and for the inside-out membranes 137 and 264 μ mol $\cdot$ mg chl⁻¹ $\cdot$ h⁻¹ in the absence and presence of diphenyl carbazide, respectively.

part of their water splitting activity is lost, which explains why diphenyl carbazide stimulated electron transport in the control samples. In the presence of diphenyl carbazide not only a total restoration but also a small stimulation of the DCIP reduction was observed for the inside-out fraction after trypsin treatment (fig.1b). This might reflect an increased accessibility of diphenyl carbazide to the tryptic-digested surface.

4. Discussion

The isolation of inside-out vesicles allows for the first time a direct comparison of proteolytic effects on the outer and inner thylakoid surfaces. The tryptination of the inside-out vesicles resulted in loss

of photosystem II electron transport which could be fully restored by addition of diphenyl carbazide. This was not the case for thylakoid membranes of normal sidedness. These results strongly suggest that the water-splitting site is located at the inner thylakoid surface.

The absence of any effect of trypsin on the reaction from diphenyl carbazide to DCIP in the inside-out fraction indicates that this reaction sequence is not exposed to the inner surface. In fact an external location is suggested since this electron transport path is affected in rightside-out thylakoids. Whether this trypsin block is on the donor side (between diphenyl carbazide and P680) or on the acceptor side can not be judged at present. Studies are in progress to solve this question.

A minor effect on the water-splitting reaction of trypsin from the outside can not be excluded since a small stimulatory effect of diphenyl carbazide was found for the rightside-out material. This stimulation might be due to secondary effects as suggested [18].

For the unbroken chloroplast lamellae the effects of trypsin on the reaction from water to DCIP were similar to those reported [8,9]. The small stimulation on addition of an artificial electron donor for photosystem II, such as diphenyl carbazide or tetraphenyl boron, is in agreement with [8,10] but not with [9] where total reactivation was reported.

Concerning the tryptic effect on the water-splitting activity from the inner surface, our results contradict [9]. On sonication of *Chlamydomonas reinhardtii* chloroplast vesicles in the presence of trypsin, followed by addition of trypsin inhibitor, they observed no deleterious effects on the water-splitting system or any other electron transport activities measured. In our opinion it is difficult to compare the effect of trypsin when, on one hand, it is added externally and on the other hand, trapped inside vesicles, since it is hard to control the amount actually trapped. Therefore, direct comparison between inside-out and rightside-out membranes is a preferable approach.

In conclusion, our findings argue strongly for a model where the water-splitting system is exposed at the inner thylakoid surface, while an external location is suggested for a part of the pathway between diphenyl carbazide and DCIP.

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References

- [1] Trebst, A. (1974) *Ann. Rev. Plant. Physiol.* 25, 423–458.
- [2] Diner, B. A. and Joliot, P. (1977) in: *Encyclopedia of Plant Physiology* (Trebst, A. and Avron, M. eds) vol. 5, pp. 187–205, Springer-Verlag, Berlin.
- [3] Fowler, C. F. and Kok, B. (1974) *Biochim. Biophys. Acta* 357, 299–307.
- [4] Ausländer, W. and Junge, W. (1975) *FEBS Lett.* 59, 310–315.
- [5] Blankenship, R. E. and Sauer, K. (1974) *Biochim. Biophys. Acta* 357, 252–266.
- [6] Giaquinta, R. T., Dilley, R. A. and Anderson, B. J. (1973) *Biochim. Biophys. Res. Commun.* 52, 1410–1417.
- [7] Arntzen, C. J., Vernotte, C., Briantais, J. M. and Armond, P. (1974) *Biochim. Biophys. Acta* 368, 39–53.
- [8] Selman, B. R. and Bannister, T. T. (1971) *Biochim. Biophys. Acta* 253, 428–436.
- [9] Regitz, G. and Ohad, I. (1976) *J. Biol. Chem.* 251, 247–252.
- [10] Renger, G., Erixon, K., Döring, G. and Wolff, Ch. (1976) *Biochim. Biophys. Acta* 440, 278–286.
- [11] Renger, G. (1976) *Biochim. Biophys. Acta* 440, 287–300.
- [12] Andersson, B., Åkerlund, H.-E. and Albertsson, P.-Å. (1977) *FEBS Lett.* 77, 141–145.
- [13] Andersson, B. and Åkerlund, H.-E. (1978) *Biochim. Biophys. Acta* 503, 462–472.
- [14] Gräber, P., Zickler, A. and Åkerlund, H.-E. (1978) *FEBS Lett.* 96, 233–237.
- [15] Andersson, B., Simpson, D. J. and Høyer-Hansen, G. (1978) *Carlsberg Res. Commun.* 43, 77–89.
- [16] Åkerlund, H.-E., Andersson, B. and Albertsson, P.-Å. (1976) *Biochim. Biophys. Acta* 449, 525–535.
- [17] Johansson, G., Hartman, A. and Albertsson, P.-Å. (1973) *Eur. J. Biochem.* 33, 379–386.
- [18] Renger, G. and Tieman, R. (1979) *Biochim. Biophys. Acta* 545, 316–324.

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LOCALIZATION OF A 34 000 AND A 23 000 M_r POLYPEPTIDE TO THE LUMENAL SIDE OF THE THYLAKOID MEMBRANE

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1. Introduction

Several attempts have been made to localize different components across the thylakoid membrane. The methods used include treatments with membrane-impermeable agents such as antibodies [1], chemical modifiers [2,3] and proteolytic enzymes [3]. This has given valuable information about the outer surface of the thylakoid membrane and has revealed that components like ferredoxin, ferredoxin-NADP-reductase, the coupling factor and the light-harvesting complex are all accessible from the outside. Information about the inner thylakoid surface is poor however and the localization of components on this side has often been deduced from indirect measurements. A direct study of the inner side has become possible after the isolation of inside-out thylakoids [4–9].

In [10] trypsination of inside-out thylakoid vesicles was used to demonstrate that at least a part of the water-splitting system is exposed to the lumen.

Here, inside-out thylakoid vesicles have been treated with trypsin or alkaline-Tris. The subsequent changes in the polypeptide pattern were followed by SDS-PAGE.

2. Materials and methods

2.1. Preparation of thylakoid membrane fractions

Stacked spinach thylakoid membranes [11] were fragmented by a Yeda press disintegration procedure

Abbreviations: chl, chlorophyll; Tris, tris(hydroxymethyl) aminoethane; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; M_r , relative molecular mass

[4]. Inside-out thylakoid vesicles formed during this procedure were separated from right-side-out material by partition in an aqueous dextran-polyethylene glycol two-phase system [4]. Chloroplast fragments were added to a polymer mixture to yield the following composition: 5.7% (w/w) Dextran T-500, 5.7% (w/w) polyethylene glycol 4000, 10 mmol/kg sodium phosphate buffer (pH 7.4), 5 mmol/kg NaCl, 20 mmol/kg sucrose and chloroplast material corresponding to 160–240 $\mu\text{g chl/ml}$. The phase system was carefully mixed and allowed to settle. The inside-out material, partitioning to the lower phase, was purified by repeating the partition procedure twice with pure upper phase. The purified inside-out thylakoids were removed from the polymers by dilution and centrifugation for 30 min at 100 000 $\times g$ and finally resuspended in 10 mM sodium phosphate buffer (pH 7.4) containing 5 mM NaCl and 100 mM sucrose. Destacked thylakoids were used as the right-side-out material.

2.2. Trypsin treatment

Trypsin (Sigma type XI) treatment was at 20°C with a sample concentration corresponding to 400 $\mu\text{g chl/ml}$. The digestion was stopped after 2.5 min by injection of solubilizing buffer (see below) at 80°C and kept at 80°C for 2 min.

2.3. Tris treatment

Tris treatment was carried out by incubating the membranes at a concentration corresponding to 200 $\mu\text{g chl/ml}$, in 0.8 M Tris (pH 8.0) for 30 min and 4°C. The incubation was followed by centrifugation for 30 min at 100 000 $\times g$ for inside-out thylakoids and 10 min at 2000 $\times g$ for right-side-out thylakoids.

The sediments and supernatants were collected and dialyzed against 0.1% SDS in order to remove remaining Tris. Before solubilization the sediments were set to 400 μg chl/ml while the supernatants were enriched to give a staining comparable to the sediments.

2.4. SDS-polyacrylamide gel electrophoresis

Samples were solubilized for 2 min at 80°C in a medium of the following composition: 5% (v/v) mercaptoethanol, 2% (w/v) SDS, 150 mM sucrose, 6 mM EDTA and 54 mM Tris-SO₄ buffer (pH 6.10). Electrophoresis was performed in the discontinuous buffer system [12] using slab gels with an acrylamide gradient of 13–17% (2.7% crosslinking). Electrophoresis was run for 6 h at 20°C and a constant current density of 10 mA/cm². The gels were stained with Coomassie brilliant blue R250. The M_r standards were bovine serum albumin (68 000), ovalbumin (43 000), soybean trypsin inhibitor (21 600) and myoglobin (17 200).

3. Results

3.1. Trypsin treatment

Several membrane polypeptides were degraded by trypsin in both right-side-out and inside-out thylakoid fractions (fig.1). Of special interest is a 34 000 M_r polypeptide which was degraded exclusively in the inside-out fraction, demonstrating that this polypeptide is exposed at the luminal side of the thylakoid membrane. The polypeptide pattern for treated right-side-out thylakoids reveals that polypeptides with app. M_r 25 000 and 26 000–27 000, probably representing apoproteins of the light-harvesting complex, were almost quantitatively degraded. These results for right-side-out thylakoid membranes are in good agreement with [13,14]. These polypeptides were also slightly affected in the inside-out fraction. This effect varied between different preparations and may to some extent be due to contamination by right-side-out material in the inside-out fraction or to limited penetration of the enzyme.

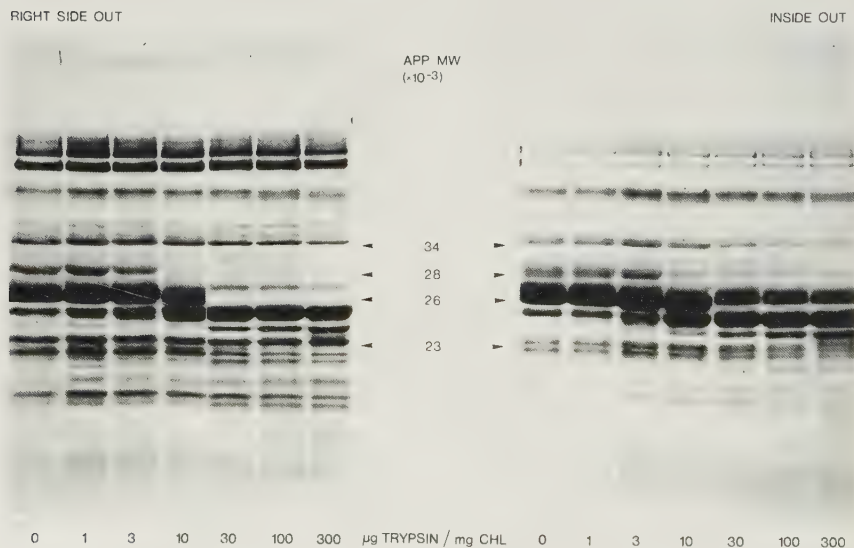


Fig.1. Effects of trypsin on the polypeptide pattern for thylakoids of opposite sidedness.

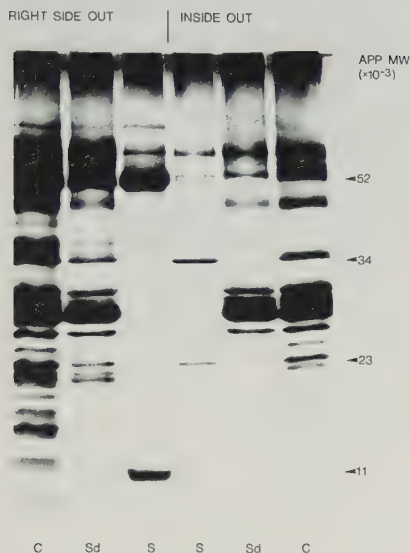


Fig. 2. Effects of Tris washing on the polypeptide pattern for thylakoids of opposite sidedness: C, control; Sd, sediment after Tris washing; S, supernatant after Tris washing.

3.2. Tris treatment

Alkaline Tris washing removed several polypeptides from the thylakoids (fig. 2). From the right-side-out material there were only two polypeptides that were quantitatively removed. One in the 52 000–53 000 M_r region represents the large subunit of ribulose 1,5-bisphosphate carboxylase (or possibly the β -subunit of the coupling factor). The other, with app. M_r 11 000, is presumably the small subunit of the carboxylase.

For the inside-out fraction, two polypeptides with app. M_r 34 000 and 23 000 were completely removed from the thylakoids by Tris washing and recovered in the supernatant. In contrast to the right-side-out material, these polypeptides were found in the sediment fraction.

4. Discussion

The trypsin and Tris treatments demonstrated

independently that a 34 000 M_r polypeptide was exposed to the surrounding medium in the inside-out but not in the right-side-out thylakoid fraction. Furthermore, the Tris treatment also removed a 23 000 M_r polypeptide specifically from the inside-out thylakoids. These two polypeptides are therefore suggested to be extrinsic and located at the inner surface of the native thylakoid membrane.

Several observations suggest that the 34 000 M_r polypeptide is a part of photosystem II. In [15] a 34 000 M_r polypeptide was enriched in photosystem II fractions but almost completely absent in photosystem I fractions. In [16,17] mutants, of *Scenedesmus* and *Zea mays*, respectively, showed a total or partial loss of a polypeptide in the 32 000–34 000 M_r region. The loss of this polypeptide was correlated with a loss of water-splitting capacity [16] while the reaction centre activity of photosystem II seemed to be unaffected [16,17]. In [18] a 33 000 M_r polypeptide has been isolated, characterized and ascribed a function in photosystem II.

In [10], mild trypsination, under conditions similar to those described here, severely inhibited the water splitting activity in inside-out thylakoids, while the right-side-out thylakoids were only slightly affected. The clear effect of trypsin on the 34 000 M_r polypeptide specifically in inside-out thylakoids (fig. 1) and the ascribed function of this polypeptide in the water-splitting system [16] suggest that the trypsin effect on the water-splitting reaction in inside-out thylakoids [10] is due to the degradation of this 34 000 M_r polypeptide.

It is interesting to note that treatments with as high as 2 M NaCl quantitatively release the 23 000 M_r but not the 34 000 M_r polypeptide from the inside-out thylakoids [19]. This suggests that the release of the 34 000 M_r polypeptide upon Tris treatment has a more specific cause than just charge shielding. One known effect of alkaline-Tris is the extraction of Mn^{2+} [20,21]. If this implies that Mn^{2+} is involved in the binding of the 34 000 M_r polypeptide to the membrane or if the release of the polypeptide and Mn^{2+} are independent effects of Tris treatment is an open question.

In conclusion, two polypeptides with app. M_r 34 000 and 23 000 have been found to be exposed on the luminal side of the thylakoid membrane. Furthermore, the 34 000 M_r polypeptide is probably associated with the water-splitting complex.

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References

- [1] Berzborn, R. J. and Lockau, W. (1977) in: *Encyclopedia of Plant Physiology* (Trebst, A. and Avron, M. eds) pp. 283–296, Springer-Verlag, Berlin, New York.
- [2] Arntzen, C. J., Vernotte, C., Briantais, J. M. and Armond, P. (1974) *Biochim. Biophys. Acta* 368, 39–53.
- [3] Renger, G. (1976) *Biochim. Biophys. Acta* 440, 287–300.
- [4] Andersson, B. and Akerlund, H.-E. (1978) *Biochim. Biophys. Acta* 503, 462–472.
- [5] Andersson, B., Akerlund, H.-E. and Albertsson, P.-Å. (1980) in *Proc. 5th Int. Congr. Photosynthesis*, in press.
- [6] Andersson, B., Akerlund, H.-E. and Albertsson, P.-Å. (1977) *FEBS Lett.* 77, 141–145.
- [7] Gräber, P., Zickler, A. and Akerlund, H.-E. (1978) *FEBS Lett.* 96, 233–237.
- [8] Andersson, B., Simpson, D. J. and Høyer-Hansen, G. (1973) *Carlsberg Res. Commun.* 43, 77–89.
- [9] Andersson, B., Sundby, C. and Albertsson, P.-Å. (1980) *Biochim. Biophys. Acta* 599, 391–402.
- [10] Jansson, Ch., Andersson, B. and Akerlund, H.-E. (1979) *FEBS Lett.* 105, 177–180.
- [11] Giaquinta, R. T., Dilley, R. A. and Anderson, B. J. (1973) *Biochem. Biophys. Res. Commun.* 52, 1410–1417.
- [12] Neville, D. M. (1971) *J. Biol. Chem.* 246, 6328–6334.
- [13] Steinback, K., Burke, J. J. and Arntzen, C. J. (1979) *Arch. Biochem. Biophys.* 195, 546–557.
- [14] Carter, D. and Staehelin, L. A. (1980) *Arch. Biochem. Biophys.* 200, 364–373.
- [15] Novak-Hofer, I. and Siegenthaler, P.-A. (1977) *Biochim. Biophys. Acta* 468, 461–471.
- [16] Metz, J. C., Wong, J. and Bishop, N. I. (1980) *FEBS Lett.* 114, 61–66.
- [17] Leto, K. J. and Miles, D. (1980) *Plant Physiol.* 66, 18–24.
- [18] Kuwabara, T. and Murata, N. (1979) *Biochim. Biophys. Acta* 581, 228–236.
- [19] Akerlund, H.-E. (1981) in: *Proc. 5th Int. Cong. Photosynthesis* in press.
- [20] Blankenship, R. E., Babcock, G. T. and Sauer, K. (1974) *Biochim. Biophys. Acta* 387, 165–175.
- [21] Cheniae, G. M. and Martin, I. F. (1978) *Biochim. Biophys. Acta* 502, 321–344.

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RECONSTITUTION OF PHOTOSYNTHETIC WATER SPLITTING IN INSIDE-OUT THYLAKOID VESICLES AND IDENTIFICATION OF A PARTICIPATING POLYPEPTIDE *

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Key words: Electron transport; Photosynthesis; Oxygen evolution; Water splitting; Thylakoid polypeptide; (Spinach chloroplast)

Inside-out spinach thylakoid vesicles have been used for inhibition and reconstitution of photosynthetic water splitting. Washing inside-out vesicles with a buffer containing 250 mM NaCl inhibited 75% of the Photosystem II reduction of phenyl-*p*-benzoquinone. In contrast, the same treatment of right-side-out vesicles gave quite a small inhibition. The site of inhibition was shown by fluorescence induction to be located at the water-splitting side of P-680. The salt-induced inhibition was accompanied by the release of two polypeptides (23 and 16 kDa) from the inner thylakoid surface. Readdition of the salt-washed supernatant or a crude chloroplast extract to the washed inside-out vesicles at low ionic strength gave a 2.7-fold stimulation of the water-splitting activity, thereby restoring about 60% of the activity lost by salt washing. This stimulation was abolished by proteolysis of the reconstituting fractions. Restoration of the water-splitting reaction was accompanied by rebinding of the 23 and 16 kDa polypeptides to the inside-out vesicles. After polypeptide purification by ion-exchange chromatography it was shown that the 23 kDa polypeptide was responsible for the restorative effect. These observations provide strong evidence that the 23 kDa polypeptide, electrostatically bound to the inner thylakoid surface, is involved in the photosynthetic water-splitting reaction.

Introduction

Photosynthetic water splitting is a fundamental process shown by all green plants and algae, including the cyanobacteria. The mechanism of water splitting is still far from understood despite intense research. There is, however, a considerable amount of kinetic information available [1,2] and the basic scheme for charge accumulation proposed by Kok et al. [3] has become widely accepted.

In contrast, information about protein components involved in the water-splitting reaction is very limited. Some polypeptides have, however,

been suggested from circumstantial evidence. Metz et al. [4] reported the lack of a 34 kDa polypeptide from a *Scenedesmus* mutant specifically deprived of water-splitting capacity and with a low manganese-to-chlorophyll ratio. Åkerlund and Jansson [5] used mild trypsinization to digest a 34 kDa polypeptide specifically in inside-out thylakoids, a treatment shown earlier to inhibit the water-splitting activity [6]. They also demonstrated [5] release of 34 and 23 kDa polypeptides specifically from inside-out thylakoid vesicles by washing with alkaline Tris, known to inhibit water oxidation [7]. Later, Yamamoto et al. [8] reported the release of 33, 24 and 18 kDa polypeptides from oxygen-evolving PS II detergent particles on Tris washing.

* A preliminary report on part of this work has been published [16].

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; PS, photosystem; Chl, chlorophyll; Me₂SO, dimethyl sulfoxide.

Reconstitution obtained with purified polypeptides should be a direct way to identify water-splitting components. Some early studies using this approach [9–11] do not seem to have been confirmed or extended. More recently, Spector and Winget [12] reported the isolation of a 65 kDa polypeptide able to reconstitute water-splitting activity in cholate-extracted thylakoid membranes incorporated into liposomes. This protein contained manganese and was claimed to be the site for Tris action in thylakoids. Later, Nakatani et al. [13] following the same preparation procedure obtained a similar protein. However, this protein contained iron instead of manganese.

For direct reconstitution experiments on the water-splitting system, the use of inside-out thylakoid vesicles [14] should be advantageous. These vesicles retain high rates of oxygen evolution [15] and expose the water-splitting enzyme system to the surrounding medium [6]. Therefore, it should be possible to reach and to affect the components of the water-splitting system without the use of detergents or sonication. Thus, it should be possible to release any weakly bound water-splitting components by mild treatments without gross damage of the thylakoid membrane. This makes the conditions for reconstitution favorable, since the subsequent readdition of the released components to the native inner thylakoid surface is possible. In a preliminary study by Åkerlund [16] this approach was shown to be promising.

In the present study, the water-splitting activity of inside-out thylakoids was inhibited by washing with 250 mM NaCl and reconstituted by readdition of the released proteins. Two main polypeptides, with apparent molecular weights of 23000 and 16000, were released by the salt treatment and could be rebound to the membrane at low ionic strength. The 23 kDa polypeptide was shown by fractionation studies to be the component responsible for reconstitution.

Materials and Methods

Poly(ethylene glycol) 4000 (Carbowax 3350) was obtained from Union Carbide, New York, NY, U.S.A., and dextran T 500, batch No. 2836, from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Preparation of inside-out thylakoid vesicles

Inside-out thylakoid vesicles were prepared according to a modification of an earlier procedure [14]. Spinach leaves were homogenized in a medium composed of 300 mM sucrose, 50 mM sodium phosphate buffer (pH 7.4) and 10 mM $MgCl_2$. The slurry was filtered through four layers of nylon mesh (25 μm) and centrifuged ($1000 \times g$, 1 min). The chloroplasts were resuspended in the same medium, pelleted by centrifugation ($1000 \times g$, 10 min) and hypotonically shocked in 5 mM $MgCl_2$. The broken chloroplasts obtained were washed three times by centrifugation ($2000 \times g$, 5 min) in 300 mM sucrose, 10 mM Tricine buffer (pH 7.4) and 5 mM $MgCl_2$ and finally suspended in 100 mM sucrose, 10 mM sodium phosphate buffer (pH 7.4), 5 mM NaCl and 5 mM $MgCl_2$. The stacked thylakoids were disintegrated twice in a Yeda press at a nitrogen pressure of 10 MPa. To attain further disintegration, EDTA to a final concentration of 5 mM was added followed by two more press treatments. After a low-speed centrifugation ($1000 \times g$, 10 min) to remove starch grains and unbroken lamellae, the inside-out thylakoid vesicles formed during the disintegration procedure [17] were separated from right-side-out material by partition in an aqueous dextran/poly(ethylene glycol) two-phase system [14]. 1 ml of the Yeda press homogenate was added to 24 g of a polymer mixture to yield the following composition: 5.7% (w/w) dextran T 500, 5.7% (w/w) poly(ethylene glycol) 4000, 10 mmol/kg sodium phosphate buffer (pH 7.4), 5 mmol/kg NaCl, 20 mmol/kg sucrose and thylakoid material corresponding to 4 mg Chl. The phase system was thoroughly mixed and allowed to settle. The inside-out material, partitioning to the lower phase, was purified by repeating the partition procedure twice with pure upper phase. The purified inside-out vesicles were freed from polymers by dilution (two to four times) in 500 mM sucrose, 5 mM sodium phosphate buffer (pH 7.4) and 2.5 mM NaCl, followed by centrifugation ($100000 \times g$, 30 min). The vesicles were resuspended in 500 mM sucrose, 5 mM sodium phosphate buffer (pH 7.4) and 2.5 mM NaCl to a concentration of 500 μg Chl/ml. Part of the material was made 5% with respect to Me_2SO and stored in liquid nitrogen. The remaining material

was diluted to 200 μg Chl/ml and used for salt treatment. Broken chloroplasts were used as right-side-out material.

Salt washing of inside-out thylakoids

The inside-out thylakoid vesicles (200 μg Chl/ml) were diluted ten times with a high-salt medium composed of 10 mM sodium phosphate buffer (pH 7.4), 250 mM NaCl and 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride and incubated on ice for 30 min. After centrifugation ($100000 \times g$, 30 min) the membranes were suspended in 500 mM sucrose, 5 mM sodium phosphate buffer (pH 7.4), 2.5 mM NaCl and 5% Me_2SO to a concentration of 500 μg Chl/ml and stored in liquid nitrogen. The supernatant was set aside for further processing.

Preparations of reconstituting fractions

Two alternative fractions were prepared.

(a) The supernatant of the salt-washed inside-out vesicles was concentrated with respect to protein by ultrafiltration (Amicon PM 10 Diaflo^R membrane, cutoff 10 kDa) and desalted to less than 5 mM NaCl by repeated dilutions and filtrations. This fraction is referred to as the salt-wash supernatant.

(b) Broken chloroplasts isolated from 2.5 kg spinach were lipid depleted with 90% acetone (pre-cooled to -20°C) for 20 min. This was repeated once and the second sediment was pelleted by centrifugation ($10000 \times g$, 10 min) and allowed to dry. 10 g of dry powder were homogenized with 300 ml water and incubated at 4°C for 1 h with stirring. The suspension was centrifuged ($10000 \times g$, 10 min) and the supernatant was collected. This supernatant is referred to as the crude chloroplast extract.

A small portion of the crude chloroplast extract was incubated on ice for 10 min with subtilisin (Sigma, protease type VIII). The subtilisin/protein ratio (w/w) was 0.2. The proteolytic activity was stopped by addition of 1 mM phenylmethylsulfonyl fluoride (freshly made in ethanol).

The crude chloroplast extract was fractionated on a DEAE-Sephacel^R (Pharmacia Fine Chemicals) column. 20 ml of sample were diluted to 60 ml with 10 mM sodium phosphate buffer (pH 6.2) and 50 mM NaCl and loaded on the column. The two fractions were eluted with 10 mM sodium

phosphate buffer (pH 6.2) and 50 mM NaCl and the remaining fractions with a linear NaCl gradient, 50–1000 mM in the same buffer. The fractions were desalted and concentrated as described above.

Protein determination was made according to the method of Bearden [18], adopted to suit dual-wavelength instrumentation.

Addition of reconstituting fractions to salt-washed inside-out thylakoids

The various protein fractions were added to washed inside-out thylakoid vesicles under low ionic strength and analysed for restorative effect on oxygen evolution and fluorescence induction. The incubation medium was composed of 70 mM sucrose, 30 mM sodium phosphate buffer (pH 6.5), 3 mM NaCl and thylakoid material corresponding to 20 and 7.2 μg Chl/ml for measurements of oxygen evolution and fluorescence, respectively. For analyses of rebinding the thylakoid vesicles were freed from the incubation medium and suspended in 500 mM sucrose, 5 mM sodium phosphate buffer (pH 7.4), 2.5 mM NaCl and 5% Me_2SO to a concentration of 400 μg Chl/ml and stored in liquid nitrogen.

Measurements of water-splitting activity

The oxygen evolution with phenyl-*p*-benzoquinone as PS II electron acceptor [19] was followed using a Clark-type oxygen electrode at 20°C . Continuous illumination was provided by a slide projector giving a quantum flux density of 2000 $\mu\text{E}/\text{m}^2$ per s between 400 and 700 nm. The measurements were performed in the incubation medium (see above) supplemented with 0.2 mM phenyl-*p*-benzoquinone. The thylakoid material corresponded to 20 μg Chl and protein fractions were added as indicated. The final volume was adjusted to 1 ml with water.

Fluorescence measurements

Room-temperature fluorescence was measured with continuous excitation light provided by the actinic source at 90° to the photomultiplier of an Aminco DW-2 spectrophotometer. The excitation light was passed through a 452 nm interference filter (half bandwidth 76 nm). The incident quantum density was 133 $\mu\text{E}/\text{m}^2$ per s. The emitted

fluorescence was filtered through a 682 nm interference filter (half bandwidth 11 nm). The photomultiplier output was amplified and recorded with a Nicolet 527 signal averager. The assay was performed in the incubation medium (see above) with the thylakoid material corresponding to 7.2 μg Chl/ml. Where indicated NH_2OH (final concentration 6 mM) or reconstituting fractions were added. The final volume was adjusted to 3 ml with water. For each measurement a new sample was dark adapted for 20 min.

SDS-polyacrylamide gel electrophoresis

Prior to electrophoresis, membrane fractions were centrifuged ($100000 \times g$, 30 min) and suspended in water to a concentration of 400 μg Chl/ml. Membrane and protein samples were solubilized for 3 min at 80°C in a medium of the following composition: 5% (v/v) mercaptoethanol, 1% (w/v) SDS, 10% (w/v) glycerol and 62 mM Tris-HCl buffer (pH 6.81). Electrophoresis was performed in the buffer system of Laemmli [20], using slab gels with an acrylamide gradient of 12–20% (2.7% cross-linking). Electrophoresis was run for 19 h at a constant current density of 4 mA/cm^2 . The gels were stained with Coomassie brilliant blue R 250. Molecular weight standards were bovine serum albumin (68000), ovalbumin (45000), carbonic anhydrase (30000), soybean

trypsin inhibitor (22000), myoglobin (17000), ribonuclease (14000) and cytochrome *c* (12000).

Results

High-salt treatment of biological membranes is frequently used for the release of electrostatically bound components, like many peripheral proteins. Table I shows the effect on PS II electron transport after washing thylakoid membranes of opposite sidedness with a buffer containing 250 mM NaCl. The activity was measured as oxygen evolution under relatively low ionic strength, using the PS II acceptor phenyl-*p*-benzoquinone after suspension of the thylakoid membranes in a low-salt buffer. After the high-salt washing as much as 75% of the PS II activity was lost in the inside-out thylakoid vesicles while only 15% of the original activity was lost in thylakoids of normal sidedness (Table I). Such inhibition and reconstitution have been obtained in numerous experiments although the absolute values have varied. The same inhibitory effects were obtained by washing with 250 mM LiCl or KCl (not shown). (The higher control rate for the everted vesicles compared to right-side-out thylakoids reflects their PS II enrichment [15,21].)

Since the salt should easily penetrate the membrane during the 30 min incubation, the pronounced difference in inhibition between inside-out

TABLE I

INHIBITION AND RECONSTITUTION OF PS II OXYGEN EVOLUTION IN INSIDE-OUT THYLAKOID VESICLES

PS II activity from water to phenyl-*p*-benzoquinone was measured as oxygen evolution with a Clark-type electrode. Inside-out and right-side-out thylakoids (400 μg Chl/ml) were washed with a high-salt buffer (pH 7.4) containing 250 mM NaCl and assayed for activity under low-salt conditions before and after addition of the reconstituting fractions. The amount of protein added during reconstitution corresponded to 1.5 and 2.0 μg protein/ μg Chl for the salt-wash supernatant and crude chloroplast extract, respectively

Material	Oxygen evolution ($\mu\text{mol O}_2/\text{mg Chl per h}$)	Inhibition (%)	Stimulation (%)	Reconstitution (%)
Inside-out thylakoids				
Control	153			
Salt washed	40	74		
Salt washed + salt-wash supernatant	109		172	61
Salt washed + chloroplast extract	107		168	59
Control + chloroplast extract	170		11	
Right-side-out thylakoids				
Control	128			
Salt washed	109	15		
Salt washed + chloroplast extract	86		0	0

and normal thylakoids should not be due to accessibility restrictions but may be explained as illustrated in Fig. 1. Under high-salt conditions components are detached from the membrane in both right-sided and inside-out material. In the inside-out vesicles, components released from the original inner thylakoid surface are lost to the supernatant during centrifugation, while for the right-sided material these components are retained in the luminal space and able to reassociate with the membrane after resuspension in a low-salt medium. This reversibility also requires that the released components have not been denatured by the treatment. (The lack of total inhibition in the inside-out fraction on salt treatment may be due to the contamination of right-side-out vesicles [22].)

The considerations above suggest that the supernatant after salt washing of inside-out thylakoids would contain active components able to restore the PS II-mediated oxygen evolution. This was tested by readdition of the concentrated salt-wash supernatant to the washed inside-out vesicles under low ionic strength. As shown in Table I, this led to an about 2.7-fold stimulation of the oxygen evolution, thereby restoring more than 60% of the activity lost due to salt washing.

Processing of the reconstituting salt-wash supernatant in terms of desalting and concentration was found cumbersome and gave quite a low recovery and therefore an alternative way for obtaining the reconstituting components was tried. Large amounts of chloroplasts were isolated followed by lipid depletion with acetone. Since the components responsible for reconstitution apparently are water soluble, the acetone powder was extracted with water. Such a crude chloroplast extract was added back to the washed inside-out thylakoids. Table I shows that this led to a stimulation of the oxygen evolution comparable to that obtained for the salt-wash supernatant. It should be pointed out that a high stimulation was obtained only for salt-washed inside-out thylakoids. For right-sided thylakoids or unwashed inside-out thylakoids there was no or only a small stimulation.

These observations (Table I) suggest that specific components involved in the electron transport through PS II were reversibly detached from the inner thylakoid surface. By only measuring the oxygen evolution associated with reduction of a PS

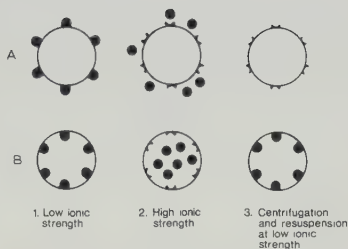


Fig. 1. Schematic representation of salt-dependent release and reassociation of components after washing thylakoid membranes of opposite sidedness. (A) Inside-out, (B) right-side-out.

II acceptor, one cannot judge whether the inhibitory and stimulatory effects are on the water-splitting or reducing side of the reaction center P-680 [6,23]. Since the water splitting complex is exposed at the inner thylakoid surface [6], it may be suggested that the reconstituting components released from this surface are involved in the water-splitting reaction. In order to confirm this, fluorescence induction was studied for the various thylakoids. Inhibitors on the water-splitting side of P-680 are known to reduce the variable fluorescence and also to give a slower rise time [24]. In contrast, inhibitors on the reducing side, like in the case of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), result in a more rapid rise of the variable fluorescence [24]. As shown in Fig. 2, the fluorescence induction curve for inside-out thylakoid vesicles after salt treatment shows a decrease in amplitude but a more pronounced increase in the rise time compared to the untreated material. Accordingly, this suggests that the inhibition site was on the water-splitting side. This is further supported by the result obtained after the addition of NH_2OH , a PS II donor [25] which restored the rapid rise time of the variable fluorescence of the washed inside-out vesicles. Faster rise times were also obtained after addition of the salt-wash supernatant ($t_{1/2} = 0.30$ s) and the crude chloroplast extract ($t_{1/2} = 0.25$ s), demonstrating restorative effects. Taken together with the effects on the electron-transport rates, these fluorescence measurements give strong evidence that the inhibitory and reconstituting effects are indeed exerted on the water-splitting side of P-680.

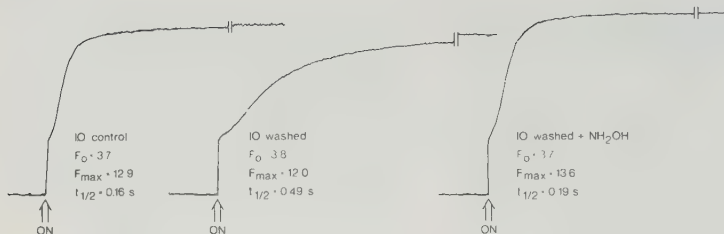


Fig. 2. Fluorescence induction curves for unwashed and salt washed inside-out (I.O.) thylakoids. Bar represents 1 s in the first part and 10 s in the second part of each trace.

In order to elucidate the nature of the reconstituting component(s), the crude chloroplast extract was treated with the proteolytic enzyme subtilisin before addition to the thylakoids. This treatment almost completely abolished the stimulating power of the extract, thereby strongly suggesting that the restoring components are proteins.

In an attempt to identify the protein components involved in this reversible inhibition of the oxygen evolution, SDS-polyacrylamide gel electrophoresis analyses were applied to the various fractions during the inhibition and reactivation experiments. Comparison of the polypeptide composition of control inside-out vesicles with that of the salt washed inside-out vesicles (Fig. 3a and b) shows that the salt released mainly two polypeptides with apparent molecular weights of 23000 and 16000 while other polypeptides were mainly unaffected. As expected, these two polypeptides were the major constituents of the salt-wash supernatant (Fig. 3e). This also contained a major band at 21 kDa which apparently was not released from the membrane upon washing and may therefore be a degradation product of the 23 kDa polypeptide. Salt treatment of right-sided thylakoids releases mainly subunits from the coupling factor [16]. The polypeptide pattern of the crude chloroplast extract is shown in Fig. 3d. As expected from the way of preparation, it contained a lot of polypeptides, including the 23 and 16 kDa polypeptides. By readdition of this chloroplast extract under low ionic strength to the washed inside-out vesicles followed by centrifugation, the binding of polypeptides to the original inner thylakoid surface

was studied. Fig. 3c shows that pronounced binding was seen only for the 23 and 16 kDa polypeptides. This points to quite a specific binding.

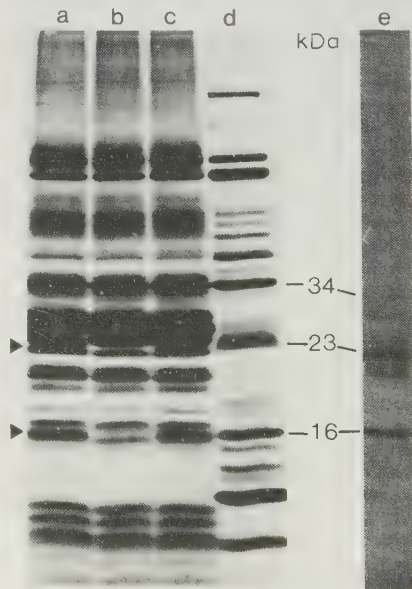


Fig. 3. Polypeptide pattern for inside-out thylakoid vesicles (a) Control, (b) salt washed, (c) salt washed after binding experiment with crude chloroplast extract, (d) crude chloroplast extract, (e) salt-wash supernatant. The arrows indicate the 23 and 16 kDa bands.

since the chloroplast extract contained numerous polypeptides, some in quite high amounts, which showed no or very little binding. Only two other polypeptides with apparent molecular weights of 62000 and 10000 showed some binding. Since they were not released from the membrane by the salt washing, their involvement in the inhibitory and restorative effects on the oxygen evolution is unlikely. Readdition of the concentrated salt-wash supernatant to washed inside-out vesicles under low ionic strength also led to rebinding of the 23 and 16 kDa polypeptides (not shown).

As shown in Table II, the stimulatory effect on oxygen evolution remained even after spinning the membranes down, following incubation with the protein fractions under low ionic strength. This result shows that the restoration of oxygen evolution was not due only to the presence of polypeptides but also to rebinding of the 23 and 16 kDa polypeptides to the original inner thylakoid surface. This gives further support to one or both of these polypeptides being responsible for the reconstitution of the oxygen evolution.

In order to purify the active polypeptide(s), ion-exchange chromatography on DEAE-Sephacel was performed. To obtain sufficient amounts of protein for this fractionation the crude chloroplast extract had to be used rather than the less complex salt-wash supernatant. The elution pattern in Fig. 4 shows several peaks from which fractions 1–9 were

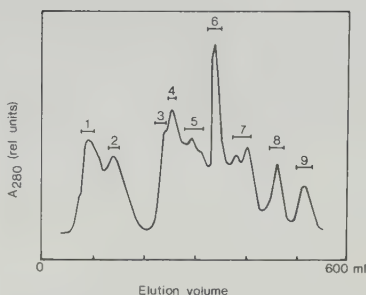


Fig. 4. Elution profile from ion-exchange chromatography on DEAE-Sephacel of the crude chloroplast extract. Fractions were collected as indicated.

collected. For each fraction, tubes were pooled followed by concentration and desalting. Fig. 5 compares the stimulatory effect on oxygen evolution of two of these protein fractions to that of the unfractionated crude chloroplast extract. Only for fraction 2 was an improved stimulation obtained, with half-maximal stimulation at 50 μg protein/mg Chl compared to 300 μg /mg for the unfractionated material. Moreover, fraction 2 reached about the same maximal stimulation compared to the chloroplast extract. All other fractions gave a quite low stimulation, comparable to or less than that of fraction 1 (Fig. 5).

TABLE II

RECONSTITUTION OF OXYGEN EVOLUTION BY REBINDING OF POLYPEPTIDES TO THE INNER THYLAKOID SURFACE

PS II activity from water to phenyl-*p*-benzoquinone was measured as oxygen evolution with a Clark-type electrode. Salt-washed inside-out thylakoid vesicles were incubated with the salt-wash supernatant or the crude chloroplast extract at low ionic strength. The amount of protein added during incubation corresponded to 1.5 and 2.0 μg protein/ μg Chl for the salt-wash supernatant and crude chloroplast extract, respectively. The vesicles were spun down in order to remove unbound components and assayed for activity under low-salt conditions.

Material	Oxygen evolution ($\mu\text{mol O}_2/\text{mg Chl per h}$)	Stimulation (%)
Inside-out thylakoids		
Salt washed	50	
Salt washed after binding experiment		
with the salt-wash supernatant	121	142
Salt washed after binding experiment		
with the chloroplast extract	133	158

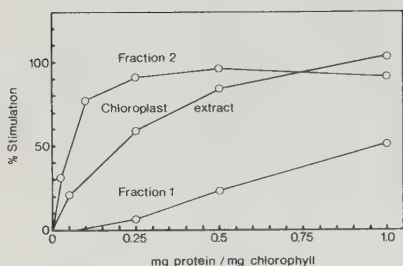


Fig. 5. Stimulation curves for oxygen evolution of salt-washed inside-out thylakoid vesicles obtained by addition of the crude chloroplast extract or fractions 1 or 2 from the chromatographic fractionation. Other fractions gave a stimulation comparable to or less than that of fraction 1.

Fig. 6 shows the polypeptides of the different fractions obtained after chromatography. The stimulating fraction 2 contained the 23 kDa polypeptide with very small amounts of other polypeptides. The 16 kDa polypeptide was the major band of fraction 1, which showed a rather poor stimulation. In the fractions that exhibited such a low stimulating capacity as in the case of fractions

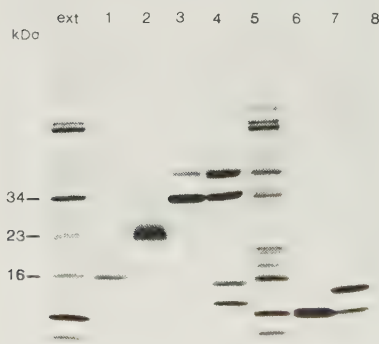


Fig. 6. Polypeptide pattern for the fractions obtained after ion-exchange chromatography of the crude chloroplast extract. 1–8, fraction numbers; ext, crude chloroplast extract.

1, 3 and 4, this could be attributed to the presence of small amounts of the 23 kDa polypeptide (Fig. 6). The amount of fraction 2 protein needed for half-maximal stimulation was 50 μ g protein/mg Chl. Assuming a Chl/P-680 ratio of 300 for the inside-out thylakoids, taking their PS II enrichment into account, the number of 23 kDa polypeptides per reaction center was calculated to be 1.3. This stoichiometry is reasonable for a component involved in photosynthetic water splitting. These calculations makes the participation of any minor polypeptides of peak 2 (Fig. 6) unlikely, since their amount compared to P-680 would be far too low.

Discussion

By inhibition and restoration studies on inside-out thylakoid vesicles, we have demonstrated the involvement of a 23 kDa polypeptide in photosynthetic water splitting. That the reconstitution was specific and not due to some general effects of the assay conditions was concluded from the following observations. (a) Inhibition and restoration of the water-splitting activity was seen for inside-out thylakoids but not for right-side-out thylakoids. (b) The restorative capacity of the reconstituting fractions was abolished by proteolysis. (c) Reconstitution was accompanied by rebinding of specific polypeptides (23 and 16 kDa) to the inner thylakoid surface. (d) Only one polypeptide (23 kDa) out of several could stimulate.

The detailed properties of the stimulating 23 kDa polypeptide have not yet been established but the following characteristics are known. It is a water-soluble peripheral protein electrostatically bound to the inner thylakoid surface. This is suggested by its salt-dependent release from and reassociation with the inside-out vesicles without any need for detergents. Its weak binding to the ion-exchanger DEAE-Sephacel indicates that the protein is only slightly negative or even positively charged at neutral pH. The polypeptide is active after being kept frozen for several months. The polypeptide gives a fuzzy band in SDS-polyacrylamide gel electrophoresis, which may indicate the involvement of intrachain sulfhydryl groups that are hard to keep reduced throughout the electrophoretic run. Of special interest is whether

or not the 23 kDa polypeptide contains manganese. This metal is reversibly released by Tris washing [26] and has been ascribed a central role in the charge accumulation necessary for oxygen evolution [27]. The metal composition of the polypeptide is currently being investigated and preliminary analyses show no manganese associated with the polypeptide.

Apart from the 23 kDa polypeptide, a 16 kDa polypeptide was released from the inner thylakoid surface by high-salt washing and rebound under low-salt conditions. Moreover, this polypeptide is enriched in PS II fractions (not shown). However, when purified, this 16 kDa polypeptide could not contribute to any stimulation. Another polypeptide apparently lacking reconstituting effect was the 34 kDa polypeptide, highly purified in fraction 3 after chromatography (Fig. 6). Still, this polypeptide is completely released from inside-out thylakoids by Tris washing [5], a treatment known to cause inhibition of oxygen evolution [7]. Thus, circumstantial evidence supports the involvement of both the 16 and 34 kDa polypeptides in photosynthetic water splitting. This is also supported by recent results by other groups [4,8,28]. At present, the possibility cannot be excluded that these two polypeptides lost their activity during the isolation procedure. This may explain why total restoration of the lost water splitting was not obtained. Together with the 16 and 34 kDa polypeptides, the active 23 kDa polypeptide may be part of a multi-enzyme complex. Together with intrinsic polypeptides, these three water-soluble components may constitute a membrane-spanning complex in analogy with the mitochondrial cytochrome *c* oxidase [29].

There are a few early reports in the literature of components able to stimulate water-splitting activity. Fredricks and Jagendorf [9] reported a component, apparently a protein, which could stimulate the PS II activity in blue-green algae, but according to their own interpretation this component was not likely to be involved on the water-splitting side. Tel-Or and Avron [10] described a manganese-containing component from blue-green algae. This factor could reconstitute the PS II activity in hypotonically washed spheroplasts from *Phormidium luridum*. The site of reactivation was inferred to be on the water-splitting side. However,

the heat stability and low molecular weight of this component make it unlikely as a polypeptide. Huzisige et al. [11] reported a factor derived from Tris-treated chloroplasts on sonication. This factor could stimulate the PS II activity in untreated but not in Tris-washed grana fractions. Although these works [9–11] were quite early, there have been no confirmational or extended studies presented. The different 65 kDa polypeptides more recently reported by Spector and Winget [12] and Nakatani et al. [13] could both restore water-splitting capacity in cholate-extracted thylakoid membranes, incorporated into liposomes. Cheniae and Sayre [30] could reconstitute the oxygen evolution in cholate-extracted thylakoids with components from the cholate supernatant. Since the 65 kDa polypeptides apparently are water soluble, the need for detergents and artificial membrane systems during isolation and reconstitution may reflect the internal location of the water-splitting components in the thylakoids. This explains why detergents did not have to be applied in the present study, where inside-out thylakoid vesicles were used. Whether there is any relation between our 23 kDa polypeptide and the proteins obtained by Nakatani et al. [13] and Spector and Winget [12] has yet to be established.

An obvious question is in what way our isolated 23 kDa polypeptide reconstitutes oxygen evolution. There are at least two possible alternatives. The first and perhaps the most straightforward explanation is that the protein is directly involved in the electron transport from water to $P-680^+$ as an electron carrier. The estimated 23 kDa polypeptide/ $P-680$ ratio of 1.3 seems to support such a conclusion. The other possibility is that the isolated protein in some way regulates the activity of the water-splitting enzyme, possibly by influencing the deactivation of S-states. Further investigations have to be made to distinguish between these alternatives.

The weak binding of the protein to the membrane may suggest that it can be free in the intrathylakoid space under certain conditions, e.g., at high ionic strength. This is in accordance with a model proposed by Lavorel [31] to explain the damping of oscillation in oxygen yield under flashing light conditions. In this model it is suggested that at least a part of the water-splitting complex

is soluble in the intrathylakoid space. The recent results of Maison-Peteri et al. [32] indeed show that the damping of oscillation in oxygen yield measured on intact thylakoids was affected by the ionic composition of the medium: at pH 7.5 an increase in damping was observed on going from 100 mM KCl to 20 mM MgCl₂.

The present isolation of a protein able to reconstitute water splitting in inside-out thylakoids offers unique possibilities to study this process. Studies are in progress to investigate further the properties of the 23 kDa polypeptide and to ascribe to it a more specific function in the water-splitting complex.

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References

- 1 Renger, G. and Eckert, H.J. (1980) *Bioelectrochem. Bioenerg.* 7, 101–124
- 2 Bouges-Bocquet, B. (1980) *Biochim. Biophys. Acta* 594, 85–103
- 3 Kok, B., Forbush, B. and McGloin, M. (1970) *Photochem Photobiol.* 11, 457–475
- 4 Metz, J.G., Wong, J. and Bishop, N.I. (1980) *FEBS Lett.* 114, 61–66
- 5 Åkerlund, H.-E. and Jansson, C. (1981) *FEBS Lett.* 124, 229–232
- 6 Jansson, C., Andersson, B. and Åkerlund, H.-E. (1979) *FEBS Lett.* 105, 177–180
- 7 Cheniae, G.M. and Martin, I.F. (1978) *Biochim. Biophys. Acta* 502, 321–344
- 8 Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) *FEBS Lett.* 133, 265–268
- 9 Fredricks, W. and Jagendorf, A. (1964) *Arch. Biochem. Biophys.* 104, 39–49
- 10 Tel-Or, E. and Avron, M. (1975) in *Proceedings of the 3rd International Congress on Photosynthesis* (Avron, M., ed.), pp. 569–578, Elsevier, Amsterdam
- 11 Huzisige, H., Isimoto, M. and Inoue, H. (1968) in *Comparative Biochemistry and Biophysics of Photosynthesis* (Shibata, K., ed.), pp. 170–178, University of Tokyo Press, Tokyo
- 12 Spector, M. and Winget, G.D. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 957–959
- 13 Nakatani, H.Y., Barber, J. and Evans, M.C.W. (1981) in *Proceedings of the 5th International Congress on Photosynthesis* (Akoyunoglou, G., ed.), vol. 2, pp. 487–494, Balaban International Science Services, Philadelphia, PA
- 14 Andersson, B. and Åkerlund, H.-E. (1978) *Biochim. Biophys. Acta* 503, 462–472
- 15 Åkerlund, H.-E., Andersson, B. and Albertsson, P.-Å. (1976) *Biochim. Biophys. Acta* 449, 525–535
- 16 Åkerlund, H.-E. (1981) in *Proceedings of the 5th International Congress on Photosynthesis* (Akoyunoglou, G., ed.), vol. 2, pp. 465–472, Balaban International Science Services, Philadelphia, PA
- 17 Andersson, B., Sundby, C. and Albertsson, P.-Å. (1980) *Biochim. Biophys. Acta* 599, 391–402
- 18 Bearden, J.C., Jr. (1978) *Biochim. Biophys. Acta* 533, 525–529
- 19 Andersson, B., Åkerlund, H.-E. and Albertsson, P.-Å. (1977) *FEBS Lett.* 77, 141–145
- 20 Laemmli, U.K. (1970) *Nature* 227, 680–685
- 21 Andersson, B. and Anderson, J.M. (1980) *Biochim. Biophys. Acta* 593, 427–440
- 22 Andersson, B., Simpson, D.J. and Høyer-Hansen, G. (1978) *Carlsberg Res. Commun.* 43, 77–89
- 23 Trebst, A. (1980) *Methods Enzymol.* 69, 675–715
- 24 Papageorgiou, G. (1975) in *Bioenergetics of Photosynthesis* (Govindjee, ed.), pp. 319–371, Academic Press, New York
- 25 Bennoun, P. and Joliot, A. (1969) *Biochim. Biophys. Acta* 189, 85–94
- 26 Blankenship, R.E., Babcock, G.T. and Sauer, K. (1975) *Biochim. Biophys. Acta* 387, 165–175
- 27 Wydrzynski, T. and Sauer, K. (1980) *Biochim. Biophys. Acta* 589, 56–70
- 28 Henry, L.E.A. and Lindberg-Møller, B. (1981) *Carlsberg Res. Commun.* 46, 227–242
- 29 Eytan, G.D., Carrol, R.C., Schatz, G. and Racker, E. (1975) *J. Biol. Chem.* 250, 8598–8603
- 30 Cheniae, G. and Sayre, R. (1981) in *Proceedings of the 5th International Congress on Photosynthesis* (Akoyunoglou, G., ed.), vol. 2, pp. 473–485, Balaban International Science Services, Philadelphia, PA
- 31 Lavorel, J. (1976) *FEBS Lett.* 66, 164–167
- 32 Maison-Peteri, B., Vernotte, C. and Baintais, J.-M. (1981) *Biochim. Biophys. Acta* 637, 202–208

IV

Isolation and characterization of a 23 kDa protein essential for photosynthetic oxygen evolution

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Abstract. A 23 kDa protein has recently been demonstrated to participate in photosynthetic oxygen evolution by reconstitution experiments on inside-out thylakoid vesicles (Åkerlund H-E, Jansson C and Andersson B (1982) *Biochim Biophys Acta* 681:1–10). Here we describe the isolation of the 23 kDa protein from a spinach chloroplast extract using ion-exchange chromatography. The protein was obtained in a yield of 25% and with less than 1% of contaminating proteins. The ability of the protein to stimulate oxygen evolution in inside-out thylakoids was preserved throughout the various fractionation steps. The isolated protein was highly water soluble and appeared as a monomer. Its isoelectric point was at $pH = 7.3$. The amino acid composition showed a high content of polar amino acids, resulting in a polarity index of 49%. The isolated protein lacked metals and other prosthetic groups. Its function as a catalytic or regulating subunit in the oxygen evolving complex is discussed.

Introduction

The polypeptide composition of the oxygen evolving complex has remained an unsolved problem but quite recent studies suggest the participation of three polypeptides of approximately 33, 23 and 16 kDa [1, 2, 3, 8, 11, 19, 21]. Various treatments that inactivate oxygen evolution in inside-out thylakoid vesicles [1, 2, 3] or in photosystem II particles prepared by detergents [8, 21] showed release or modification of these three polypeptides. In contrast to such circumstantial evidence, removal and reconstitution experiments would more directly demonstrate the involvement of a particular polypeptide in oxygen evolution. Recently we succeeded in such an approach [3]. The oxygen evolution of inside-out thylakoid vesicles was inhibited by a salt-wash treatment concomitant with a release of the 23 kDa protein from the membrane. Readdition of this polypeptide, contained in a crude chloroplast extract or in a more purified form, to the washed inside-out vesicles, restored up to 60% of the lost activity. The restoration was accompanied by a rebinding of the 23 kDa protein to the inner thylakoid surface. These results clearly demonstrated the involvement of the 23 kDa protein in the oxygen evolving system.

Abbreviations: kDa, kiloDalton; PAGE, polyacrylamide gel electrophoresis.

In the present study we describe the isolation and characterization of this 23 kDa protein essential for photosynthetic oxygen evolution.

Materials and methods

Purification of the 23 kDa protein

Step 1. Broken chloroplasts from 2.5 kg of spinach leaves were prepared as previously described [3] and suspended in water to a concentration of 4 mg chlorophyll $\cdot$ ml⁻¹. To this suspension nine volumes of acetone, precooled to -20°C, was added with continuous stirring. After 20 min of stirring, at an ambient temperature of 4°C, the suspension was allowed to settle and the supernatant was discarded. The procedure was repeated once and the second sediment was pelleted by centrifugation (10,000 $\cdot$ g, 10 min). The pellet was smeared out, pulverized and air-dried until the powder was free from acetone. Each gram of dry powder was mixed with 10 ml of 10 mM sodium phosphate pH 6.4, 50 mM NaCl and 2 mM of the protease inhibitor, phenylmethylsulfonylfluoride. The slurry was homogenized in a glass homogenizer using a motor-driven pestle and was incubated for 1 hr at 4°C with stirring. After centrifugation (10,000 $\cdot$ g, 10 min) the supernatant (designated crude protein extract) was collected.

Step 2. 100 ml of the crude protein extract was applied to a DEAE-Sephacel[®] column (35 cm $\cdot$ 4.5 cm²) preequilibrated with 10 mM sodium phosphate pH 6.4, 50 mM NaCl. The elution was performed with 10 mM sodium phosphate pH 6.4, 50 mM NaCl. The flow rate was 10 cm $\cdot$ hr⁻¹ (52 ml $\cdot$ hr⁻¹). The first 400 ml of the protein-containing eluate was collected and desalted by repetitive concentrations and dilutions with 10 mM sodium phosphate pH 6.6 using a YM 10 Diaflo[®] ultrafiltration membrane.

Step 3. The desalted eluate was applied to a CM Sepharose[®] CL-6B column (22 cm $\cdot$ 2.0 cm²) preequilibrated with 10 mM sodium phosphate pH 6.6 and the 23 kDa protein was eluted with a 0-400 mM NaCl gradient in the same buffer. The flow rate was 10 cm $\cdot$ hr⁻¹ (25 ml $\cdot$ hr⁻¹). The 23 kDa protein appeared in the first main peak. The fractions were collected and desalted as described above.

Step 4. For further purification, step 3 was repeated once. Protein concentration was determined according to [4].

Characterizations of the 23 kDa protein

SDS-PAGE was performed as previously described [3] in the buffer system of Laemmli [9]. The gels were stained with Coomassie Brilliant blue. The purity of the protein fractions was estimated from densitograms of the stained gels.

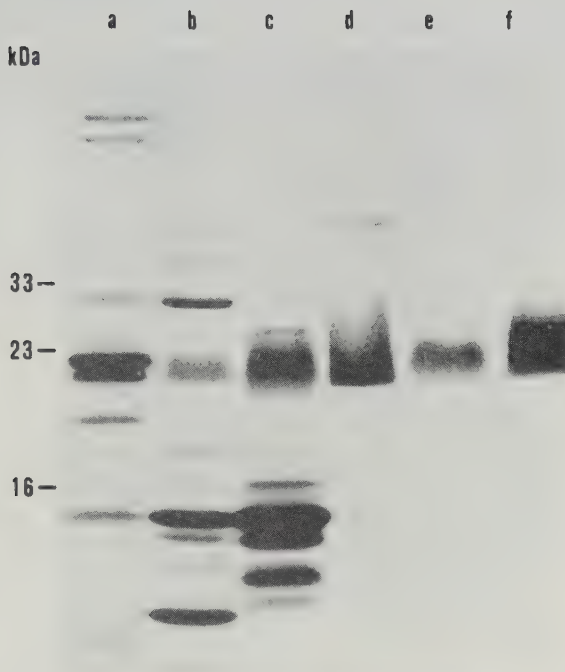


Figure 1. SDS-PAGE of various fractions from the purification of the 23 kDa protein. a, broken chloroplasts; b, crude protein extract (step 1); c, DEAE-Sephacel preparation (step 2); d, 1st CM-Sephadex preparation (step 3); e and f, 2nd CM-Sephadex preparation (step 4). The amounts of protein applied was 60 μ g (slots a–d), 30 μ g (slot e) and 200 μ g (slot f). The location of the bands corresponding to 33, 23 and 16 kDa are indicated.

Isoelectric focusing was performed according to [12] except that Triton and urea were omitted.

The molecular weight was determined by gel filtration on a Sephadex[®] G-100 column (90 cm $\times$ 2.0 cm²).

For amino acid analysis the 23 kDa protein was hydrolyzed in 6 M HCl for 24 and 72 hr. The analysis was performed with a Durrum D-500 amino acid analyser. Cysteine was determined as half-cystine after oxidation with performic acid. Proline was determined with cysteine oxidized. The values for threonine and serine were obtained after extrapolation to zero time hydrolysis. Tryptophan was determined after oxidation with mercaptoethanesulfonic acid [13]. For metal analysis particle induced X-ray emission (PIXE) analysis was performed mainly as described in [7].

The capacity for the various protein fractions to restore oxygen evolution to salt-treated inside-out thylakoid was followed by using a Hansatech oxygen

Table 1. Purification of the 23 kDa protein from spinach chloroplasts. The stimulatory capacity was estimated as the inverse value of the amount of protein needed to obtain half maximum stimulation of the oxygen evolution in salt-washed inside-out thylakoid vesicles

Preparation	Yield (%)	Purity (%)	Stimulatory capacity (μg^{-1} protein $\cdot$ μg chlorophyll)
Crude protein extract (step 1)	100	17	5
DEAE Sephacel (step 2)	74	26	
1st CM Sepharose (step 3)	33	90	
2nd CM Sepharose (step 4)	25	> 99	25

electrode [3]. The inside-out thylakoid vesicles were isolated by phase partition and washed in 250 mM NaCl as described earlier [3].

Results

The 23 kDa protein was purified from a crude spinach chloroplast protein extract by ion-exchange chromatography. The crude protein extract was obtained by water extraction of chloroplasts delipidated by acetone, and contained some 20 polypeptides as revealed by SDS-PAGE (Figure 1 slot b). The 23 kDa polypeptide constituted about 17% of the total protein content as judged by Coomassie Brilliant blue staining (Figure 1 slot b, Table 1). By this step hydrophobic membrane proteins such as the apopolypeptides of the light harvesting complex (25, 23 kDa) could be removed (Figure 1 slots a and b). The crude protein extract was fractionated by DEAE-Sephacel chromatography. The 23 kDa polypeptide was eluted together with the 16 kDa and some other polypeptides (Figure 1 slot c). The majority of the polypeptides, including the 33 kDa polypeptide, was retained on the column.

The 23 kDa polypeptide was separated from the 16 kDa polypeptide by fractionation on a CM Sepharose column. Upon elution with a NaCl gradient (0–400 mM) the 23 kDa protein appeared in the first main fraction, between NaCl concentrations of 50 and 150 mM (Figure 1 slot d). The CM Sepharose step was repeated once to remove other polypeptides giving less than 1% contamination as judged by Coomassie staining on an overloaded gel (Figure 1 slot f). The high purity of this final preparation was mostly reproducible but in case of less pure preparations they were subjected to still another CM Sepharose step. As shown in Table 1, the yield in the final preparation was 25% on the basis of the crude protein extract. Typically, around 5 mg of the pure protein was obtained from 2.5 kg of spinach leaves.

Table 1 also compares the capacity of the pure protein and the crude protein extract with respect to their stimulatory effect on oxygen evolution in salt-washed inside-out thylakoids. The rate of oxygen evolution in control inside-out vesicles, $168 \mu\text{mol O}_2 \cdot \text{mg Chl}^{-1} \cdot \text{h}^{-1}$, dropped to $47 \mu\text{mol O}_2 \cdot \text{mg Chl}^{-1} \cdot \text{h}^{-1}$ after salt-washing in accordance with previous observations [3]. Addition of the crude chloroplast extract or the pure 23 kDa protein gave a

Table 2. Amino acid composition of the 23 kDa protein^a

Amino acid	Mole %	Residues per protein (mole/mole)
Asp/Asn	11.1	23.6 (24)
Thr ^b	6.0	12.7 (13)
Ser ^b	9.0	19.2 (19)
Glu/Gln	9.2	19.7 (20)
Pro	4.5	9.6 (10)
Gly	9.7	20.7 (21)
Ala	8.0	17.1 (17)
Cys ^c	0.7	1.4 (1)
Val	8.5	18.2 (18)
Met	0.6	1.3 (1)
Ile	2.1	4.4 (4)
Leu	5.8	12.4 (12)
Tyr	4.3	9.1 (9)
Phe	6.5	13.9 (14)
His	0.6	1.2 (1)
Lys ^d	10.6	22.6 (23)
Trp ^d	0.8	1.7 (2)
Arg	1.7	3.6 (4)
Total	100	213

^a Mean values from 24 and 72 hr hydrolysis in 6 M HCl

^b Extrapolation to zero time hydrolysis

^c Determined as half-cystine after oxidation with performic acid

^d Oxidation with mercaptoethansulfonic acid [13]

The nearest integers are given within brackets

180% stimulation leading to around 60% reconstitution of the lost activity. The amount of protein needed for half maximal stimulation was $200 \mu\text{g} \cdot \text{mg}^{-1}$ chlorophyll for the crude protein extract and $40 \mu\text{g} \cdot \text{mg}^{-1}$ chlorophyll for the pure 23 kDa protein. This 5 times increase in stimulatory capacity corresponds fairly well to the 6 times increase in purity. Thus, there was very little deactivation during the fractionation procedure which means that the isolated 23 kDa protein was still fully active.

In SDS-PAGE the protein appeared with an apparent molecular weight of 23,000 (Figure 1 slot e). Typically, the stained band of the 23 kDa polypeptide was very fuzzy, particularly when the gel was overloaded. Omission of mercaptoethanol during the solubilization did not sharpen or alter the location of the band, indicating that intra- or interchain disulfide bonds are not essential for the conformation of the 23 kDa protein. The molecular weight for the undenatured protein was 25,000 as judged by gel filtration on Sephadex G-100. The similarity in molecular weight obtained by this method and that obtained by denaturing SDS-PAGE showed that the isolated protein appeared as a monomer in solution.

The amino acid composition of the 23 kDa protein given in Table 2 reveals a quite high proportion of polar amino acids, such as glutamine/glutamic acid, asparagine/aspartic acid and lysine. This results in a polarity index of 49%, which is typical for hydrophilic membrane proteins [5]. The solubility in aqueous solutions without addition of detergents showed that the polar

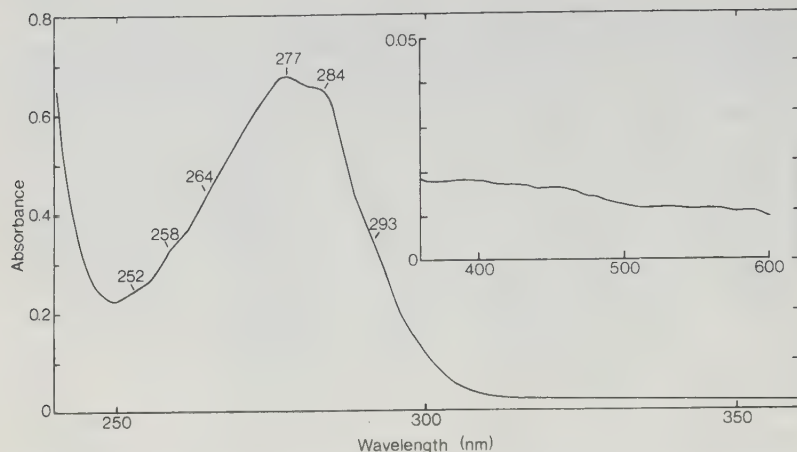


Figure 2. Absorption spectrum of the 23 kDa protein. The visible region of the spectrum is shown in inset. The protein concentration was $600 \mu\text{g} \cdot \text{ml}^{-1}$ in 10 mM sodium phosphate pH 6.6. The spectrum was recorded with an Aminco DW-2 spectrophotometer.

amino acids are exposed on the outer periphery of the protein. Isoelectric focusing showed that the protein has its isoelectric point at $\text{pH} = 7.3 \pm 0.2$.

The absorption spectrum for the 23 kDa protein is shown in Figure 2. The peak is at 277 nm where the $A_{1\text{cm}}^{1\%}$ was 10.5. The protein concentration of the sample was determined from the amino acid analysis by summing up the amounts of amino acid residues. Using the $A_{1\text{cm}}^{1\%}$ value of 10.5 and a molecular weight of 23,000 the molar extinction coefficient was calculated to be $24,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Strikingly, there is no absorption peak in the visible region of the spectrum indicative of prosthetic groups such as hemes and flavins. Moreover, no heme staining [18] of the 23 kDa polypeptide after mild SDS-PAGE could be detected. The presence of prosthetic groups absorbing in the UV region only, such as quinones, can not be excluded.

The involvement of the 23 kDa protein in oxygen evolution prompted an analysis of its metal content. This was done by a method based on Particle Induced X-ray Emission (PIXE) [7] which is a multielement analysis where most common metals such as iron, copper, manganese and zinc can be detected simultaneously. The results clearly showed that the isolated 23 kDa protein did not contain any metals. For iron, copper, manganese and zinc this was also confirmed by traditional atomic absorption measurements. It was also found that no significant amounts of metals were released from the inside-out thylakoid vesicles upon salt-washing.

The temperature stability of the 23 kDa protein was examined by following its ability to stimulate oxygen evolution activity in inside-out thylakoid vesicles, after incubation of the protein at increasing temperatures. It was found that the stimulatory capacity of the protein was mainly unaffected up to 50°C . However, at 65°C as much as 90% of its activity was lost. Since

oxygen evolution is normally inhibited at 50°C it is obvious that the 23 kDa protein is not the site for heat inactivation of oxygen evolution. The protein was fully active after several months at -20°C and could be stored for a week at 4°C without any noticeable decrease in stimulatory capacity.

Discussion

It was previously demonstrated that the 23 kDa protein is involved in the oxygen evolving system [3]. In salt-washed inside-out thylakoid vesicles the oxygen evolution activity was inhibited and the 23 kDa protein was released. Upon readdition of the salt-wash supernatant to the inhibited vesicles under low ionic strength, the oxygen evolution was partly restored, concomitant with a rebinding of the 23 kDa protein to the inner thylakoid surface. In the present study the 23 kDa protein was isolated in a pure and active form from a crude chloroplast protein mixture using ion-exchange chromatography.

The amino acid analysis revealed a high content of charged amino acids. The protein is quite water soluble without any use of detergents, suggesting that the charged amino acids are exposed on the protein surface. This is consistent with the suggestion [3] that the protein is electrostatically bound to the inner thylakoid surface as indicated by its reversible release from the membrane upon variations in the ionic strength. The release of the 23 kDa protein from the everted thylakoids was usually achieved by washing with 250 mM NaCl at pH 7.4. Since this pH is very close to the overall isoelectric point of the protein (7.3) the pKa value of the binding site should be different. This is also suggested from recent experiments which show that the 23 kDa protein can be released from inside-out thylakoid vesicles by washings at high pH (pH > 9.0) even at low ionic strength (unpublished results).

Very recently a 24 kDa polypeptide has been isolated by electrofocusing of the supernatant following Tris-washing of photosystem II particles [22]. This polypeptide share certain similarities with our protein but has its main absorption peak at 264 nm and a lower isoelectric point despite a higher content of arginine. The relation between these two proteins is difficult to judge since no data on purity or activity was reported for the 24 kDa polypeptide.

In what way the 23 kDa protein functions in the oxygen evolution is not yet clear. A protein involved in electron transport and charge accumulation associated with water oxidation would be expected to have a prosthetic group able to participate in redox reactions. The isolated 23 kDa protein, however, apparently lacks such groups. It could be argued that the native protein contains a prosthetic group which was lost during the isolation. This is not likely since the isolated protein was still active in restoring oxygen evolution. There is however a possibility that the 23 kDa protein, when rebound to the membrane, may reassociate with some cofactor present in excess amounts in the membrane, such as quinones and carotenoids. Interestingly, recent

observations have suggested that both quinones [16] and carotenoids [17] are involved in photosynthetic oxygen evolution.

In the light of the apparent lack of prosthetic groups, catalytic functions in the water splitting process other than electron transfer have to be considered for the 23 kDa protein. Such a function could be to participate in proton exchange reactions that might be necessary for charge stabilization or for efficient electron transport. Proton exchange reaction are common in enzyme catalysis and only require the participation of charged amino acids.

Another possibility is that the 23 kDa protein has a regulatory function in oxygen evolution. This would be in analogy with the mitochondrial cytochrome c oxidase, where both catalytic and regulatory subunits are known [20]. It has been shown that addition of cations can increase the damping of the oxygen yield after single turnover flashes in intact chloroplasts [10]. This observation may be connected to the release of the 23 kDa protein. The 23 kDa protein may also influence the induction of a cyclic electron transport around photosystem II. Such a cyclic electron flow can be artificially induced e.g. by Tris-treatment [6, 15] which releases the 23 kDa protein from the inner thylakoid surface [2].

A purely structural role of the 23 kDa protein can also be envisaged. In a very recent study on the interaction of amino water analogs with the O_2 evolving complex it was suggested that H_2O binds in a cleft at the thylakoid surface [14]. Such a cleft could be constituted by the 23 kDa protein alone or in combination with the 16 and 33 kDa proteins.

The possibility to obtain the 23 kDa protein in a pure and active form should be of great importance for further studies on its specific function in oxygen evolution. Its interaction with other thylakoid components may also be useful for identifying other subunits in a multicomponent oxygen evolving complex.

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References

1. Akerlund H-E (1981) in Akoyunoglou G, Ed. Proc 5th Int Cong on Photosynthesis, Vol. 2, pp 465–472. Philadelphia: Balaban International Science Services
2. Akerlund H-E and Jansson C (1981) Localization of a 34000 and a 23000 M_r polypeptide to the lumenal side of the thylakoid membrane. FEBS Lett 124: 229–232
3. Akerlund H-E, Jansson C and Andersson B (1982) Reconstitution of photosynthetic water splitting in inside out thylakoid vesicles and identification of a participating polypeptide. Biochim Biophys Acta 681:1–10

4. Bearden JC Fr (1978) Quantitation of submicrogram quantities of protein by an improved protein-dye binding assay. *Biochim Biophys Acta* 533:525–529
5. Capaldi RA and Vanderkooi G (1972) The low polarity of many membrane proteins. *Proc Natl Acad Sci USA* 69:930–932
6. Haveman J and Mathis P (1976) Flash-induced absorption changes of the primary donor of photosystem II at 820 nm in chloroplasts inhibited by low pH or Tris-treatment. *Biochim Biophys Acta* 440:346–355
7. Johansson S-A-E and Johansson TB (1976) Analytical application of Particle-Induced X-ray Emission. *Nucl Instr Meth* 137:473–516
8. Kuwabara T and Murata N (1982) Inactivation of photosynthetic oxygen evolution and concomitant release of three polypeptides in the photosystem II particles of spinach chloroplasts. *Plant and Cell Physiol* 23:533–539
9. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
10. Maison-Peteri B, Vernotte C and Briantais J-M (1981) Modifications of the functioning of photosystem II induced in the dark by alkaline pH in the presence of cations. *Biochim Biophys Acta* 637:202–208
11. Metz JG, Wong J and Bishop NI (1980) Changes in electrophoretic mobility of a chloroplast membrane polypeptide associated with the loss of the oxidizing size of photosystem II in low fluorescent mutants of *Scenedesmus*. *FEBS Lett* 114:61–66
12. Novak-Hofer I and Siegenthaler P-A (1977) Two-dimensional separation of chloroplast membrane proteins by isoelectric focusing and electrophoresis in sodium dodecyl sulphate. *Biochim Biophys Acta* 468:461–471
13. Penke B, Ferenczi R and Kovács K (1974) A new acid hydrolysis method for determining tryptophan in peptides and proteins. *Anal Biochem* 60:45–50
14. Radmer R and Ollinger O (1983) Topography of the O₂-evolving site determined with analogs *FEBS Lett* 152:39–43
15. Renger G and Eckert HJ (1981) Studies on the functional organization of photosystem II – The effect of protein-modifying procedures and of ADHY agents on the reaction pattern of photosystem II in Tris-washed chloroplasts. *Biochim Biophys Acta* 638:161–171
16. Renger G and Weiss W (1983) Spectral characterization in the ultraviolet region of the precursor or photosynthetically evolved oxygen in isolated trypsinized chloroplasts. *Biochim Biophys Acta* 722:1–11
17. Schenk CC, Diner B, Mathis P and Satoh K (1982) Flash-induced carotenoid radical cation formation in photosystem II. *Biochim Biophys Acta* 680:216–227
18. Thomas PE, Ryan D and Levin W (1976) An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Anal Biochem* 75:168–176
19. Toyoshima Y and Fukutaka E (1982) A protein essential for recovering oxygen evolution in cholate-treated chloroplasts. *FEBS Lett* 150:223–227
20. Wikström M, Krab K and Saraste M (1981) Cytochrome oxidase, a synthesis. New York: Academic Press
21. Yamamoto Y, Doi M, Tamura N and Nishimura M (1981) Release of polypeptides from highly active O₂-evolving photosystem-2 preparation by Tris treatment. *FEBS Lett* 133:265–267
22. Yamamoto Y, Shimada S and Nishimura M (1982) Purification and molecular properties of 3 polypeptides released from a highly active O₂-evolving photosystem-II preparation by Tris-treatment. *FEBS Lett* 151:49–53

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PROTEINS INVOLVED IN PHOTOSYNTHETIC OXYGEN EVOLUTION - ISOLATION AND CHARACTERIZATION

CHRISTER JANSSON

1. INTRODUCTION

Recently, much attention has been focused on three PS II proteins of approximately 33, 23 and 16 kDa and their involvement in photosynthetic oxygen evolution. For the 23 kDa protein, reconstitution experiments have provided strong evidence for a participation in the oxygen evolving complex (Åkerlund et al. 1982, Ljungberg et al. 1983, Murata et al. 1983). Also with the 16 kDa protein stimulation of oxygen evolution has been described (Toyoshima et al. 1983). Although such reconstitutions have not been reported for the 33 kDa protein, there are several lines of circumstantial evidence pointing to its involvement as a manganese protein in the oxygen evolving process. In the present study, the preparative isolation of the 33, 23 and 16 kDa proteins is described. Each protein could be obtained in a high yield and in an almost homogeneous form, as revealed by SDS-PAGE. The pure protein fractions were characterized in terms of amino acid composition, optical properties, isoelectric points and molecular weights.

2. PROCEDURE

Step 1. Broken chloroplasts from 2.5 kg spinach leaves were suspended in water to a concentration of 4 mg/ml. To this suspension nine volumes of acetone, precooled to -20°C , was added with continuous stirring. After 20 min of stirring, at an ambient temperature of 4°C , the suspension was allowed to settle and the supernatant was discarded. The procedure was repeated once and the second sediment was pelleted by centrifugation (10,000 g, 10 min). To remove the acetone, the pellet was smeared out, pulverized and air-dried. Each gram of dry powder was mixed with 10 ml of 10 mM sodium phosphate pH 6.4, 50 mM NaCl and 2 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The slurry was homogenized in a glass homogenizer using a motor-driven pestle and was incubated for 1 h at 4°C with stirring. After centrifugation (10,000 g, 10 min) the supernatant, designated the crude protein extract, was collected.

Step 2. 100 ml of the crude protein extract was applied to a DEAE-Sephacel column (35 cm 4.5 cm^2) equilibrated with 10 mM sodium phosphate pH 6.4, 50 mM NaCl. The elution was performed with 600 ml of the same medium and was pursued until no more protein was detected in the eluate. Two pools were collected. Pool 1 (400 ml) contained the bulk portion of the eluted proteins, which appeared in a large asymmetrical peak at the front of the elution - profile. Pool 2 (60 ml) contained the material from a shallow peak at the end of the elution.

Step 3. For isolation of the 33 kDa protein, pool 2 was applied to a Blue Sepharose column (15 cm 2.0 cm^2) equilibrated with 10 mM sodium phosphate pH 6.4, 50 mM NaCl. After washing with 2-3 column volumes of 10 mM sodium phosphate pH 6.4, 50 mM NaCl the 33 kDa protein was eluted with a 50-800 mM NaCl gradient in the same buffer.

Step 4. Pool 1 from step 2. was applied to a CM-Sephacel column (22 cm 2.0 cm^2) equilibrated with 10 mM sodium phosphate pH 6.4, 50 mM NaCl. The 16 kDa protein was adsorbed on the column while the 23 kDa protein was eluted with 600 ml of 10 mM sodium phosphate pH 6.4, 50 mM NaCl until no protein was

detected in the eluate. The total eluate was subsequently desalted to 10 mM sodium phosphate pH 6.4, using ultrafiltration with a YM 10 Diaflo membrane. The 16 kDa protein was eluted with 300 ml of a 50-400 mM NaCl gradient in 10 mM sodium phosphate pH 6.4.

Step 5. The eluate containing the 23 kDa protein from step 4. was applied to a second CM-Sephacrose column equilibrated with 10 mM sodium phosphate pH 6.4. After washing with 2-3 column volumes of the phosphate buffer the 23 kDa-protein was eluted with 300 ml of a 0-400 mM NaCl gradient in the same buffer. The protein appeared in the first main peak. For further purification, this 23 kDa preparation was diluted 15 times with 10 mM sodium phosphate pH 6.4, to decrease the NaCl concentration to <10 mM, and recycled on the CM-Sephacrose column.

The final protein preparations were desalted and concentrated by ultrafiltration as above.

SDS-PAGE, isoelectric focusing, amino acid and metal analyses were all performed as previously described (Jansson et al. 1983).

3. RESULTS AND DISCUSSION

The crude protein extract contained some 20 polypeptides, as revealed by SDS-PAGE (Fig.1). The 33, 23 and 16 kDa proteins constituted around 22, 17 and 25% of the total protein content respectively. In the DEAE-Sephacel chromatography step the 23 and 16 kDa proteins were eluted together in a large asymmetrical peak at the front. Separation of the 23 and 16 kDa proteins was achieved on a CM-Sephacrose column. The 16 kDa protein was adsorbed, while the 23 kDa protein was eluted. Care was taken to completely elute the 23 kDa protein from the column. The total eluate was desalted by ultrafiltration and applied to a second CM-Sephacrose column. In 10 mM sodium phosphate pH 6.4, without NaCl, the 23 kDa protein was now adsorbed and could be eluted with a 0-400 mM NaCl gradient. The 23 kDa protein appeared between 50 and 150 mM NaCl, in the first main peak. On SDS gels this preparation exhibited mainly one contaminant, at approximately 44 kDa. The nature of this polypeptide is not known. The 23 kDa preparation was diluted and subjected to another run on the CM-Sephacrose column. The dilution was preferable to desalting by ultrafiltration, since the latter resulted in a considerable loss of protein (30%). The 16 kDa protein was eluted from the first CM-Sephacrose column with a 50-400 mM NaCl gradient. The 33 kDa protein was released from the DEAE-Sephacel column upon prolonged elution with 10 mM sodium phosphate pH 6.4, 50 mM NaCl, resulting in a very shallow peak in the elution profile. It was found that these conditions minimized comigration of a 36 kDa protein, probably the ferredoxin-NADP oxido-reductase. To remove residual 36 kDa protein and other contaminants, the 33 kDa preparation was further purified on a Blue Sepharose column. The yield for each protein was 25-35%, based on the crude extract. Typically, from 100 ml of crude protein extract (corresponding to 1 kg spinach

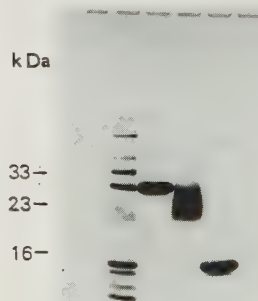


Fig.1. SDS-PAGE. In each slot 40µg protein was added. The gels were stained with Coomassie Brilliant blue. A, crude protein extract; B, 33 kDa prep; C, 23 kDa prep; D, 16 kDa prep.

leaves) 4-6 mg of each protein, more than 95% pure, was obtained.

The isolated 33, 23 and 16 kDa proteins could all rebind to their sites at the inner thylakoid surface (Larsson et al. these proceedings). The activity of the 23 kDa protein, in stimulating oxygen evolution (Åkerlund et al. 1982, Ljungberg et al. 1983) was preserved throughout the fractionation steps (Jansson et al. 1983). With the 16 kDa protein, no such reconstitution was observed (Ljungberg et al. 1983). This is in agreement with another study (Murata et al. 1983) but in contrast to results by Toyoshima et al. (1983). Thus, it could be argued that the present isolation procedure inactivates the 16

kDa protein. This is not likely, however, since the 23 and 16 kDa proteins contained in the desalted supernatant after salt-washing of inside-out thylakoid vesicles, did not show any stimulatory capacity in excess of the purified 23 kDa protein (Åkerlund et al. 1982). As for the 33 kDa protein, no successful reconstitution experiments have been reported. In accordance with other studies, we found no metals like Mn, Fe, Cu or Zn in any of the isolated proteins. Recently, however, the isolation of a Mn-containing 34 kDa protein was described (Dismukes et al. 1983). None of the isolated 33, 23 or 16 kDa proteins showed any significant absorbance in the visible region of the spectrum and no heme staining could be detected after mild SDS-PAGE. The presence of cofactors like quinones can not be excluded, although it seems unlikely, considering the acetone extraction procedure.

The isoelectric points, as determined by isoelectric focusing, was 5.2, 7.3 and 8.5 for the 33, 23 and 16 kDa proteins respectively. The amino acid compositions are given in Table 1. For all three proteins there is a high proportion of polar amino acids, resulting in polarity indices around 49%. Apparently, there is only one cysteine-residue per 23 and 16 kDa molecule, while the 33 kDa protein contains three or four. The amino acid compositions agree fairly well with those in other studies (Yamamoto et al. 1982, Murata et al. 1983).

The most striking discrepancies are the considerably higher values for Try reported by Yamamoto et al. and, in the same study, the higher content of Arg in the

TABLE 1. Amino acid compositions

Amino acid	Mole%			Residues/protein (mole/mole)		
	33	23	16	33	23	16
Asp/Asn	8.9	11.1	10.6	24	24	13
Thr	7.8	6.0	5.3	21	13	7
Ser	7.2	9.0	7.8	19	19	10
Glu/Gln	13.5	9.2	11.7	36	20	15
Pro	5.9	4.5	8.5	16	10	11
Gly	12.0	9.7	5.2	32	21	6
Ala	6.6	8.0	8.9	17	17	11
Cys	1.3	0.7	0.7	4	1	1
Val	6.7	8.5	4.4	18	18	6
Met	0.5	0.6	0.1	1	1	0
Ile	3.2	2.1	4.4	8	4	5
Leu	7.1	5.8	11.6	19	12	15
Tyr	2.8	4.3	2.6	7	9	3
Phe	5.3	6.5	2.2	14	14	3
His	0.1	0.6	0.9	0	1	1
Lys	8.3	10.6	9.3	22	23	12
Trp	0.2	0.8	0.2	1	2	0
Arg	2.4	1.7	5.4	6	4	7
Total				268	213	126
Polarity index (%)	48	49	51			

TABLE 2. Molecular properties of the proteins

	33	23	16
MW (SDS-PAGE)	33,000	23,000	16,000
Isoelectric point	5.2	7.3	8.5
Abs.max (nm)	277	277	277
ϵ (1/M,cm)	18,000	24,000	12,000

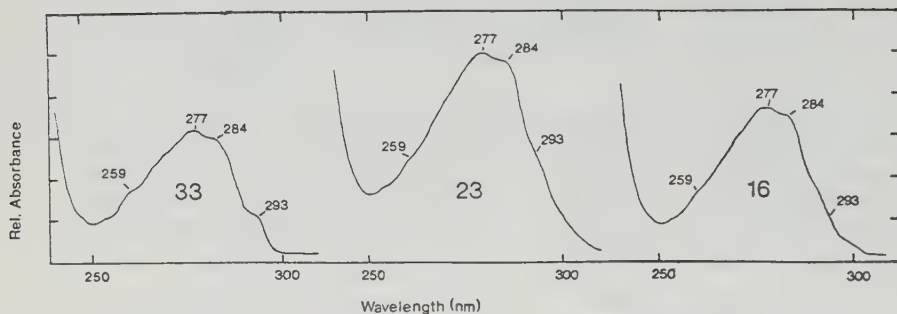


Fig.2. UV absorption spectra, recorded in 10 mM sodium phosphate pH 6.4.

23 kDa protein, despite the lower isoelectric point.

The absorption spectra in the UV region are shown in Fig.2. The absorption maxima were at 277 nm for all three proteins. Using molecular weights of 33,000, 23,000 and 16,000 and protein concentrations as determined from the amino acid analyses the molar extinction coefficients were calculated to 18,000, 24,000 and 12,000 for the 33, 23 and 16 kDa proteins respectively. Table 2. summarizes some of the molecular properties of the isolated proteins. Recently, we have shown that all three proteins are encoded by nuclear DNA and are synthesized as precursor forms (Westhoff et al. these abstracts).

REFERENCES

- Dismukes GC, Abramowicz DA, Ferris KF, Mathur P, Siderer Y, Upadrashta B and Watnick P (1983) EPR evidence for the involvement of a discrete manganese cluster in O_2 evolution. In Inoue Y ed. Oxygen-evolving System of Plant Photosynthesis. Academic Press, Tokyo. In press.
- Jansson C, Åkerlund HE and Andersson B (1983) Isolation and characterization of a 23 kDa protein essential for photosynthetic oxygen evolution. Photosynth. Res. In press.
- Ljungberg U, Jansson C, Andersson B and Åkerlund HE (1983) Reconstitution of oxygen evolution in salt-washed Photosystem II particles. Biochem. Biophys. Res. Commun. 113, 738-744.
- Murata N, Miyao M and Kuwabara T (1983) Organization of the photosynthetic oxygen evolution system. In Inoue Y ed. Oxygen-evolving System of Plant Photosynthesis. Academic Press, Tokyo. In press.
- Toyoshima Y, Akabori K, Fukutaka E and Imaoka A (1983) Proteins essential for recovering oxygen evolution in cholate-treated thylakoids. In press. Ibid.
- Yamamoto Y, Shimada S and Nishimura M (1982) Purification and molecular properties of 3 polypeptides released from a highly active O_2 -evolving photosystem II preparation by Tris-treatment. FEBS Lett. 151, 49-53.
- Åkerlund HE, Jansson C and Andersson B (1982) Reconstitution of photosynthetic water splitting in inside-out thylakoid vesicles and identification of a participating polypeptide. Biochim. Biophys. Acta 681, 1-10.

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EPR Studies on the Photosystem II Donor Side in Salt-Washed
and Reconstituted Inside-Out Thylakoids

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*Key words: photosynthetic oxygen evolution, 23 kDa protein,
inverted thylakoid vesicles, electron paramagnetic resonance,
interacting manganese ions, electron transfer*

*Abbreviations: chl, chlorophyll; PpBQ, phenyl-p-benzoquinone; PS
II, Photosystem II*

ABSTRACT

EPR measurements on inside-out thylakoids revealed that salt-washing induced a Signal II_f which was smaller than that expected from the inhibition of oxygen evolution and decreased the EPR signal from state S_2 . Readdition of the released 23 kDa protein led to a partial restoration of oxygen evolution and a decrease in Signal II_f . It is suggested that in these Photosystem II units salt-washing inhibits the electron transfer from the oxygen evolving site to Z, the physiological donor to P680. In inhibited PS II units lacking Signal II_f , Z^+ is rapidly reduced, possibly by a modified S-cycle unable to evolve oxygen.

1. INTRODUCTION

The 23 and 16 kDa PS II proteins can be specifically removed from inside-out thylakoid vesicles by salt-washing /1,2/ or from chloroplasts by deoxycholate extraction /3/, with a concomitant inhibition of oxygen evolution. Reconstitution experiments /2,4,5/ have demonstrated that readdition of the released 23 kDa protein to the salt-washed inside-out membranes could reconstitute around half of the lost activity. Fluorescence analyses /2/ and absorbance measurements on $P680^+$ reduction /6/ in connection with reversible salt-wash inhibition, showed that the functional site of the 23 kDa protein is along the electron pathway between water and P680. In the present work EPR studies were performed to investigate the reactions on the PS II donor side in salt-washed and reconstituted inside-out thylakoids.

The suggested immediate donor to $P680^+$, Z, is EPR detectable in its oxidized state. The reduction of Z^+ is highly dependent on the integrity of the oxygen-evolving complex. In unaffected thylakoids the reduction is in the submillisecond region, typical of Signal II_vf /7/. In PS II units with the oxygen evolution inhibited by e.g. Tris treatment, Z^+ is reduced slowly, with half-times between 100 ms and 1s, characteristic for Signal II_f /8,9/.

The results here show that salt-washing of inside-out thylakoids induced an increase in Signal II_f, which, however, was considerably smaller than that expected from the inhibition of oxygen evolution. The increase in Signal II_f was reversed upon readdition of the purified 23 kDa protein. In addition, the multiline EPR signal, assumed to arise from the oxidation state S_2 in the oxygen evolving complex /10-12/, was irreversibly suppressed after salt-washing.

2. MATERIALS AND METHODS

Inside-out thylakoid vesicles were prepared and then salt-washed by incubation at pH 7.4 in 250 mM NaCl as described earlier /2/. Untreated (control) and salt-washed inside-out vesicles (3.8-7.6 mg chl/ml) in 5 mM sodium phosphate (pH 7.4), 3 mM NaCl, 500 mM sucrose and 5 % dimethylsulfoxide were stored in small aliquots in liquid nitrogen until use. Reconstitution of oxygen evolution was achieved by addition of purified 23 kDa protein /4,13/ to thawed, salt-washed vesicles. Samples for low-temperature EPR were prepared as in /11/. Room-temperature EPR experiments were made in the presence of 5 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, 25 mM $MgCl_2$ and 0.1 mM EDTA /14/ with 0.3 mM PpBQ as redox mediator. After measurements, the vesicles were withdrawn from the sample cell, subjected to a Tris incubation (0.8 M Tris, pH 8.0, for 10 min at 0 °C and room light) and then reinjected into the cell for a repetition of the measurements.

EPR measurements were made with the equipment described in /11/. The room-temperature experiments were made with a Varian cylindrical cavity working in the TM 110 mode. The cavity was flushed with a precooled nitrogen gas in order to maintain the temperature in the flat sample cell (Scanlon, width 17 mm) at 20.0 °C. Flash-induced Signal II kinetics was monitored at the position of the Signal II low field peak /8/. Saturating white flashes, halfwidth 3 μ s and spaced 4 s apart, were furnished from a pulsed xenon light source (Photochemical Research Ass., Model 610 B). The flash-induced transient EPR signals (25 to 175 per sample) were computer-averaged and then fitted to a sum of one rising and one decaying exponential component. The time constant of the rising component was kept fixed at the spectrometer time constant (30 ms), while the half-time and amplitude

of the decaying component were obtained from a non-linear least-squares method. Spin concentrations were estimated by comparisons with the signal obtained from 10 mM VOSO₄ in the sample cell, using standard methods /15/. Signal II_{vf} was not observable in this work (instrument response time 30 ms).

Oxygen evolution was followed polarographically /2/ in 5 mM sodium phosphate (pH 7.4), 3 mM NaCl, 500 mM sucrose, 0.2 mM PpBQ and thylakoid material corresponding to 20 µg chl.

3. RESULTS

Dark-adapted oxygen-evolving inside-out thylakoid vesicles showed a stable EPR radical signal (Fig.1, inset) with properties typical of Signal II_u /8/ and amounting to about one spin per 250 chl. This agrees with the estimated unit size (270-340 chl/PS II) in these vesicles /16,17/. Upon illumination with brief, saturating light flashes there was a small transient increase (about 0.1 spins per PS II unit) of the Signal II amplitude (Fig.1a), measured at $g=2.011$ (Fig.1, inset), where interference from the EPR signal from P700⁺ (Signal I) is negligible. The half-time of the Signal II decay (about 300 ms) identified the transient as Signal II_f /8/, which probably represented a fraction of PS II units inactivated during preparation. In addition, an even smaller light-induced transient was seen, probably Signal II_s /8/, with a half-life of several minutes. This treatment resulted in a tenfold increase in Signal II_f with a decay half-time of about 1 s (Fig.1b, Table 1).

Salt-washing of the inside-out thylakoids, which partially inhibited the oxygen evolution and released the 23 kDa protein, resulted in an increase (0.13 spins per PS II unit) of the Signal II_f amplitude and increased the half-time of the decay to

Effects on Signal II_f by salt-washing and reconstitution of inside-out thylakoid vesicles

	O_2 -evolution (%)	Signal II_f (spins/250 chl)	$t_{1/2}$ (ms)
Control	100	0.10	332 ± 27
Control + Tris	0	1.15	866 ± 12
Salt-washed	39	0.23	701 ± 63
Salt-washed + Tris	0	0.92	1014 ± 25
Reconstituted	68	0.13	775 ± 160
Reconstituted + Tris	0	0.98	1522 ± 56

Oxygen-evolving activity was measured with PpBQ as a PS II electron acceptor. The control activity was $210 \mu\text{mol } O_2 \text{ per mg chl and h.}$ The Signal II_f measurements are described in the legend to Fig.1. After salt-washing, 40 % of the 23 kDa protein was retained in the membranes as determined by rocket immunoelectrophoresis.

about 700 ms (Fig.1c, Table 1). The other Signal II species were only slightly affected. Partial restoration of the oxygen evolution by readdition of the 23 kDa protein to the depleted membranes led to a decrease in Signal II_f amplitude whereas the decay time was unaffected (Fig.1e, Table 1). It should be mentioned, however, that the decay of these transients can be fitted equally well to a linear combination of two components with half-times of 300 ms and 1's, respectively. Tris treatment of the depleted or reconstituted inside-out thylakoids resulted in a large Signal II_f , comparable to that in Tris-treated control material (Figs.1d,f, Table 1).

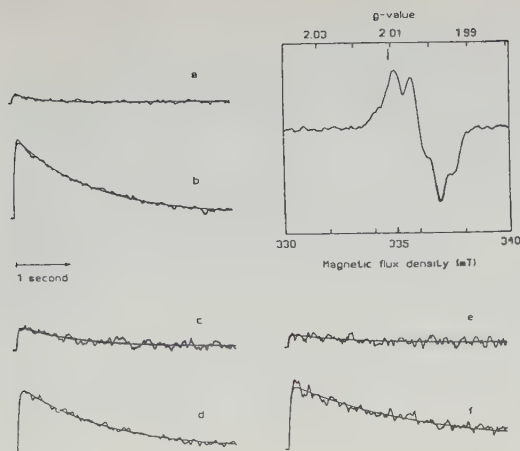


Fig.1. Flash-induced EPR signal II in inside-out thylakoids at room temperature. Trace a, flash-induced transients obtained from control inside-out thylakoids (3.6 mg chl/ml); b, sample a after Tris treatment (2.7 mg chl/ml); c, salt-washed inside-out thylakoids (3.7 mg chl/ml); d, sample c after Tris treatment (2.8 mg chl/ml); e, salt-washed thylakoids (3.2 mg chl/ml) reconstituted with the 23 kDa protein (0.9 mg protein/mg chl); f, sample e after Tris treatment (2.5 mg chl/ml). Traces are averages of 25-175 flash-induced transients. EPR conditions: microwave frequency, 9418 MHz; power, 2 mW; modulation amplitude, 0.33 mT; spectrometer time constant, 30 ms; temperature, 20.0 ± 0.5 °C. Inset, room temperature EPR spectrum of dark adapted control. EPR conditions as for a-f, but with a magnetic field scan of 0.17 mT/s and a spectrometer time constant of 0.3 s. Gain 0.4 relative to a-f. The arrow shows the magnetic field position (334.7 mT) at which the flash-induced signal II was monitored.

The control inside-out vesicles developed a multiline EPR signal when frozen in strong light (Fig.2a). The positions of the lines and their relative amplitudes were similar to those found in chloroplasts [11] and in oxygen-evolving PS II preparations (Hansson, Andréasson, unpublished observations) prepared according to Berthold et al. [18]. The amplitude of the multiline signal decreased considerably after salt treatment (to about 40 % of the control amplitude). This effect was not reversed after partial reconstitution of the oxygen evolution by readdition of the 23 kDa protein (Figs.2b and c, respectively).

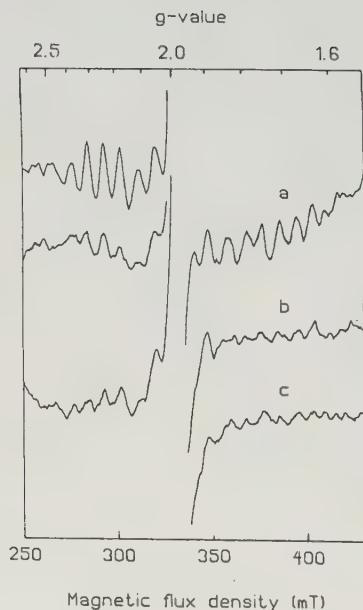


Fig.2. Low-temperature EPR spectra of illuminated inside-out thylakoids. The samples were frozen in the presence of 4 mM PpBQ and 4 mM EDTA, either in the dark or during continuous illumination. Spectrum a, control inside-out thylakoids, 6.9 mg chl/ml; b, salt-washed inside-out thylakoids, 4.9 mg chl/ml; c, salt-washed inside-out thylakoids, 4.9 mg chl/ml, after addition of purified 23 kDa protein (0.9 mg/mg chl). The spectra presented are the differences between illuminated and dark-adapted samples. EPR conditions: microwave frequency, 9227 MHz; power, 126 mW; modulation amplitude, 1.25 mT; temperature, 15 K.

4. DISCUSSION

Salt-washing of inside-out thylakoids, which inhibits the oxygen evolution through the release of the 23 kDa protein /1,2/ (Table 1), more than doubled the amplitude of Signal II_f, an increase which could be almost fully reversed by readdition of the purified 23 kDa protein. This indicates that the removal of the 23 kDa protein inhibited the electron transfer from the oxygen evolving complex to Z in a fraction of the material. It

is reasonable to assume that reconstitution of this fraction contributes to the recovery of oxygen evolution.

Considering the inhibition in oxygen evolution, the inhibited PS II centers would be expected to give rise to Signal II_f amounting to 60 % of the PS II units. The low amount actually found (10 %) suggests that Z⁺ is still rapidly reduced (faster than 30 ms, the response time of the instrument) in most of the PS II units. The component responsible for the reduction of Z⁺ might be a modified, partially functional S-cycle, incapable of oxygen evolution, or cytochrome *b*₅₅₉. In both cases a cyclic or exogenous re-reduction of this component is required. It has been shown that cytochrome *b*₅₅₉ can be rapidly oxidized by PS II /19/. However, at the redox potential (about 460 mV) in the present experiments, cytochrome *b*₅₅₉ should initially be oxidized and has to be reduced before it can deliver electrons to Z⁺, thus making this alternative less attractive.

An alternative explanation for the small Signal II_f amplitude in the salt-washed vesicles would be a complete block in the electron transfer between Z and P680, preventing the formation of Z⁺. This is less probable, however, since Tris treatment of the salt-washed vesicles or the control induced a large Signal II_f (Table 1). Other results also indicate that this segment of the electron transfer chain remains operative after salt-washing /20/.

The decrease after salt-washing in the multiline EPR signal, suggested to arise from interacting manganese ions of the S₂ state (Fig.2b) /10-12/, may be related mainly to the irreversible part of the inhibition in oxygen evolution. Under the above assumption of a modified S-cycle, the low amplitude of this signal could be explained by an inhibition in one of the

transitions leading to accumulation of the S_2 state, such as S_1-S_2 , S_3-S_4 or S_4-S_0 . Such an inhibition would still allow a limited electron transfer to Z^+ . On the other hand, if the changes in the multiline EPR signal are related to the reversible part of the inhibition in oxygen evolution, readdition of the 23 kDa protein to the salt-washed vesicles should be associated with an increase in this signal. However, as the reconstitution involves about 30 % of the total material, the expected increase could be difficult to detect.

Recent optical measurements of the decay kinetics for $P680^+$ /6/ and Q^- /21/ indicate that salt-washing of inside-out thylakoid vesicles increases the back reaction between Q^- and $P680^+$ during high repetitive (5 Hz) flash illumination. Qualitatively, these results agree with those presented here; In modified PS II units, for example those showing Signal II_f, a $P680^+-Q^-$ recombination would be expected to occur upon increasing the flash repetition rate, since the supply of electrons from Z to $P680^+$ would be rate-limited by the slow reduction of Z^+ .

Our results indicate that the removal of the 23 kDa protein may lead to deficiencies in the oxygen-evolving system such as to prevent specific S-state transitions rather than shutting off the charge-accumulating process completely. However, the S_2-S_3 transition alone is not inhibited as this should lead to an accumulation of the S_2 state and an increase in the multiline EPR signal; contrary to our observations.

After completion of this work, it was learned that the 23 kDa protein increases the affinity for chloride in the oxygen evolving complex, and that the inhibition induced by release of the 23 kDa protein can be partially relieved by addition of chloride /22/. Therefore, it cannot be excluded that the rather

high chloride conditions employed during the Signal II_f measurements may to some extent explain the small signal in the sodium chloride treated membranes. It is now important to repeat these measurements under varying chloride conditions.

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We thank professor Tore Vännngård for valuable discussions and helpful criticism and Mrs Ingun Sundén-Cullberg and Mrs Agneta Persson for skilful technical assistance. This work was sponsored by the Swedish Natural Science Research Council.

REFERENCES

- /1/ Åkerlund, H.-E. (1981) in: Photosynthesis II. Electron Transport and Photophosphorylation (Akoyunoglou, G. ed) pp. 465-472, Balaban International Science Services, Philadelphia, Pa.
- /2/ Åkerlund, H.-E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1-10.
- /3/ Franzén, L.-G. and Andréasson, L.-E. (1984) in: Proceedings of the 6th International Congress on Photosynthesis (Sybesma, C. ed) Martinus Nijhoff, The Hague. In press.
- /4/ Jansson, C., Åkerlund, H.-E. and Andersson, B. (1983) Photosynth. Res. 4, 271-279.
- /5/ Larsson, C., Jansson, C., Ljungberg, U., Åkerlund, H.-E. and Andersson, B. (1984) in: Proceedings of the 6th International Congress on Photosynthesis (Sybesma, C. ed) Martinus Nijhoff, The Hague. In press.
- /6/ Åkerlund, H.-E., Brettel, K. and Witt, H.T. (1984) Biochim. Biophys. Acta. In press.
- /7/ Blankenship, R.E., Babcock, G.T., Warden, J.T. and Sauer,

- K. (1975) FEBS Lett. 51, 287-293.
- /8/ Babcock, G.T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 329-344.
- /9/ Boska, M., Sauer, K., Buttner, W. and Babcock, G.T. (1983) Biochim. Biophys. Acta 722, 327-330.
- /10/ Dismukes, G.C. and Siderer, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 274-278.
- /11/ Hansson, Ö. and Andréasson, L.-E. (1982) Biochim. Biophys. Acta 679, 261-268.
- /12/ Andréasson, L.-E., Hansson, Ö. and Vanngård, T. (1983) Chemica Scripta 21, 71-74.
- /13/ Jansson, C. (1984) in: Proceedings of the 6th International Congress on Photosynthesis (Sybesma, C. ed) Martinus Nijhoff, The Hague. In press.
- /14/ Babcock, G.T., Ghanotakis, D.F., Ke, B. and Diner, B.A. (1983) Biochim. Biophys. Acta 723, 276-286.
- /15/ Aasa, R. and Vanngård, T. (1975) J. Magn. Reson. 19, 308-315.
- /16/ Andersson, B. and Haehnel, W. (1982) FEBS Lett. 146, 13-17.
- /17/ Anderson, J.M. and Melis, A. (1983) Proc. Natl. Acad. Sci. USA 80, 745-749.
- /18/ Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231-234.
- /19/ Velthuys, B.R. (1981) FEBS Lett. 126, 272-276.
- /20/ Renger, G. and Voelker, M. (1982) FEBS Lett. 149, 203-207.
- /21/ Åkerlund, H.-E., Renger, G., Weiss, W. and Hagemann, R. (1984) Biochim. Biophys. Acta. In press.
- /22/ Andersson, B., Critchley, C., Ryrle, I.J., Jansson, C., Larsson, C. and Anderson, J.M. (1984) FEBS Lett. In press.

VII

IMMUNOLOGICAL STUDIES ON THE ORGANISATION OF PROTEINS IN PHOTOSYNTHETIC OXYGEN EVOLUTION

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SUMMARY

Studies on inside-out thylakoid vesicles and several photosystem 2 particles have suggested the involvement of three proteins of 33, 23, and 16 kDa in photosynthetic oxygen evolution. In this study monospecific antibodies were raised against the purified 33, 23, and 16 kDa proteins. The antibodies were used to investigate the organization and function of these proteins in the oxygen evolving complex. Quantification of the 33, 23, and 16 kDa proteins by rocket immunoelectrophoresis revealed one copy of each polypeptide per some 200 chlorophylls in unfractionated thylakoids. Isolated inside-out thylakoids, derived from the grana partitions, showed 6-8 times more of the 33, 23 and 16 kDa proteins, on a chlorophyll basis, compared to the stroma lamellae vesicles. Agglutination studies revealed that the proteins are exposed on the luminal side of the thylakoid membrane. An obligatory role for the 23 kDa protein in the photosynthetic water oxidation is suggested from the close correlation obtained between the inhibition of oxygen evolution and the release of this protein as caused by washing the inside-out thylakoids with increasing concentrations of NaCl. For the 16 kDa protein no such correlation was obtained. Rebinding-experiments, using both salt-washed and alkaline Tris-washed inside-out thylakoids revealed that the 33 kDa protein was required for the binding of the 23 kDa protein which in turn enhanced the binding of the 16 kDa protein. It is concluded that the three proteins are closely organized as a complex at the inner thylakoid surface in association with membrane spanning proteins of photosystem 2.

INTRODUCTION

Recent studies on the composition of the photosynthetic oxygen evolving complex have suggested the involvement of three extrinsic polypeptides with apparent molecular weights of 33 000, 23-24000 and 16-18 000. These three polypeptides can be released from inside-out thylakoid vesicles and other oxygen evolving photosystem 2 preparations by washing with alkaline Tris or with NaCl at high concentrations, treatments that also inhibit oxygen evolution (1-4). The 23 kDa protein was shown by Åkerlund et al. (5) to partly restore oxygen evolution in salt-washed inside-out vesicles. This restorative effect on oxygen evolution by the 23 kDa protein has later been demonstrated also with other photosystem 2 preparations (6-9). Furthermore, recent studies on mutants support these findings (10). Restoration with the 16 kDa protein has so far only been reported for thylakoid particles isolated by a cholate-procedure (9). No marked restoration with the 33 kDa protein has yet been demonstrated. In mutant studies (11,12) the disappearance of a 34 kDa protein correlated with a decrease in oxygen evolving capacity and manganese content although the correspondence of this protein to the extrinsic 33 kDa protein discussed above has recently been questioned (13). The isolation of a 34 kDa protein containing manganese was recently reported (14). Thus a protein in the 33-34 kDa region is a likely candidate for the postulated mangano-protein in photosynthetic oxygen evolution. Since no prosthetic groups have so far been detected in the isolated 23 and 16 kDa proteins (7,15-18) their precise roles in oxygen evolution remain to be established.

To date the analyses of the 33, 23 and 16 kDa proteins have been confined to SDS-PAGE, which has certain limitations. Due to the complexity of the thylakoid polypeptide composition interpretations concerning one individual polypeptide can be seriously hampered by staining properties, limits of

detection and the problem of comigration. The later problem particularly concerns the 33 and 23 kDa polypeptides of oxygen evolution. The 33kDa region also contains cytochrome f (19) and two polypeptides of the photosystem 2 core complex (20) one of which is the herbicide-binding protein (21). The broad apopolypeptides of LHC-II (22) cover the 23 kDa region which also contains polypeptides of LHC-I (23) and the cytochrome b/f complex (19).

To overcome these problems we have in the present study used immunological methods for specific and sensitive analyses of the organization and function of the oxygen evolution complex. Quantification by rocket immunoelectrophoresis showed one copy of each polypeptide per some 200 chlorophylls. The three proteins are arranged in a complex at the inner thylakoid surface where the 33 kDa protein is required for the binding of the 23 kDa protein. The 23 kDa protein in turn, enhanced the binding of the 16 kDa protein. Correlation between the inhibition of oxygen evolution and release of the proteins after salt-washing of inside-out vesicles indicates an obligatory role for the 23 kDa protein but not for the 16 kDa protein.

MATERIALS AND METHODS

Isolation of proteins

The 33, 23, and 16 kDa proteins were isolated as in (18). Briefly, isolated thylakoids were treated with 90% acetone to remove lipids. The water soluble proteins were extracted, and fractionated on a DEAE-Sephacell^R column using 10 mM sodium phosphate pH 6.4, 50 mM NaCl. The 33 kDa protein was further purified by affinity chromatography using Blue Sepharose^R, while the 23 and 16 kDa proteins were purified through repetitive runs on a CM-Sepharose column, using a linear gradient of 0-400mM NaCl containing 10mM sodium phosphate pH 6.4.

Immunological methods

Each of the three isolated proteins (200-300 μ g) was mixed with one volume of Freund's complete adjuvant (Difco) and injected behind the scapulae of the rabbits. After 5 weeks the rabbits were booster-injected in the same manner except that incomplete adjuvant was used. Antisera were collected 2, 3 and 4 weeks after the booster injection. IgG was prepared from the sera by affinity chromatography on a Protein A-Sepharose CL-4B column. After elution of the bulk protein with 50 mM K_2HPO_4 , 0.5 M KCl, the IgG was eluted with 0.1 M glycine pH 3.0, dialyzed against 10 mM sodium phosphate pH 7.4, and concentrated by ultrafiltration. Control IgG was prepared from serum obtained from non-immunized rabbits. The IgG concentration was calculated from the absorbance at 280 nm, using $A_{280}=1.27$ for 1 mg IgG/ml (U.-B. Hansson, personal communication). The specificity of the antisera was determined by "Western" blotting(24) Thylakoid polypeptides were separated by SDS-PAGE and then transferred to a nitrocellulose membrane by electrophoresis. The blot was cut into strips; one strip was stained with Amido Black and the other incubated sequentially with one antiserum (0.5-5 μ l serum/10 ml medium) and peroxidase-conjugated swine antirabbit IgG. The peroxidase-containing bands were developed by incubating the strips with H_2O_2 and o-dianisidine. Rocket immunoelectrophoresis(25) was run in 0.8% agarose containing 20 mM barbital buffer pH 8.6, 0.6% Zwittergent TM-314 (Calbiochem) and 0.4% Triton X-100. 20 mM barbital buffer was used as electrode buffer. In quantification of the 16 kDa protein the pH of the barbital buffer was lowered to 7.0 since the protein has a high isoelectric point 18 so that cathodic rockets were obtained. The electrophoresis was run at 200 V at 5°C for 18 hrs. After electrophoresis the plates were washed, dried and finally stained with Coomassie brilliant blue. The amount of each antigen was determined from the rocket area. Before

electrophoresis, thylakoid membranes were solubilized in a 3% detergent mixture of Triton X-100 and Zwittergent TM-314 (2:3). The purified proteins were treated in the same way and used as standards.

Agglutination of thylakoid vesicles was investigated by mixing 10 μg of IgG (1-4 mg/ml) and 10 μg of vesicle suspension (100 μg chl/ml) at 20°C. The aggregation was followed using a phase contrast microscope and was completed within 5 min.

Preparation and treatments of inside-out thylakoid vesicles

Inside-out and right-side out thylakoid vesicles were prepared by phase partition after Yeda press treatment in the presence of 5 mM MgCl_2 as previously described (5,26). In some experiments, inside-out vesicles with a particularly high photosystem 2 enrichment and photosystem 1-rich stroma lamellae were prepared as in (27). Inside-out vesicles were washed in NaCl solutions of increasing concentrations (10-300 mM, pH 7.4) or in 100 mM Tris pH 9.0, and finally suspended in 500 mM sucrose, 5 mM sodium phosphate pH 7.4, 2.5 mM NaCl.

Rebinding of the purified proteins to the inner thylakoid surface was studied by adding 20 μg of purified protein to washed inside-out vesicles (100 μg chlorophyll) suspended in 5 ml of 70 mM sucrose, 30 mM sodium phosphate pH 6.5, 3 mM NaCl. After incubation for 30 min on ice the vesicles were sedimented at 100,000 x g for 30 min. In the case of rebinding of more than one type of protein, each addition was followed by the incubation and centrifugation steps to allow for a sequential rebinding.

Oxygen evolution with phenyl-p-benzoquinone as photosystem 2 acceptor was followed in 1 ml of a medium composed of 70 mM sucrose, 30 mM sodium phosphate pH 6.5, 3 mM NaCl and thylakoids corresponding to 20 μg chlorophyll. Chlorophyll was determined according to Arnon (28) and protein according to Bearden (29).

RESULTS

In order to obtain monospecific antibodies, the individual 33, 23 and 16 kDa proteins were purified by ion-exchange and affinity chromatography (17,18). The purity and properties of the isolated proteins have been given elsewhere (17,18). When injected into rabbits each protein gave rise to a monospecific antiserum. The monospecificity of the antisera was shown by "Western"-blotting using dissociated, intact thylakoids (Fig. 1). Each antiserum gave rise to one band at the expected apparent molecular weight. Also, in rocket immuno-electrophoresis with thylakoids each antiserum gave one main precipitation arc only.

In order to establish the location of the 23 and 16 kDa proteins in the thylakoid membrane, agglutination of inside-out and right-side out vesicles were compared. For that purpose the IgG fraction of each antiserum was used

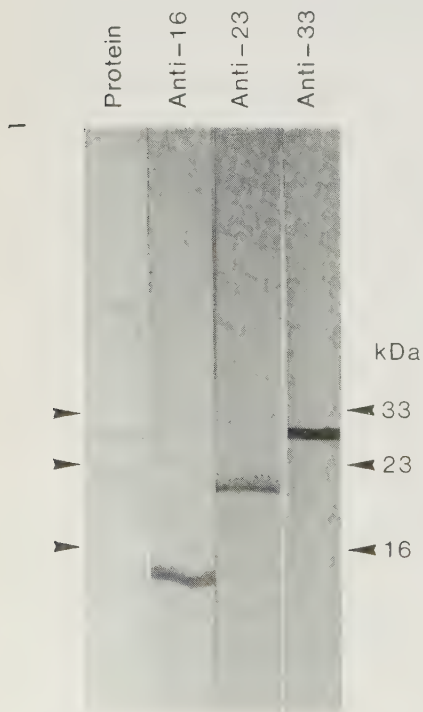


Fig.1. The specificity of sera used for immunoelectrophoresis as demonstrated by Western blotting. Thylakoid polypeptides were separated by SDS-PAGE and then blotted to a nitrocellulose membrane. The concentrations of the antisera against the 16, 23, and 33 kDa proteins were 5, 0.5, and 5 μ l/10 ml medium, respectively. Thylakoid polypeptides reacting with IgG in the sera were specifically stained

to minimize unspecific agglutination caused by the bulk serum proteins. As can be seen in Fig. 2 the IgG against the 23 and 16 kDa proteins caused agglutination of inside-out thylakoids, clearly distinguishable from the background aggregation seen with the control IgG. In contrast, the agglutination of right-side out vesicles was weak with both anti-IgG and control-IgG. This shows that the 23 and 16 kDa proteins are exposed on the inner thylakoid surface which is in accordance with the release-pattern upon salt-washing (15). The same agglutination results have previously been observed using antibodies to the 33 kDa protein (B. Andersson, L. Klein-Hitpass, C. Jansson and R. Berzborn, unpublished observations). None of the antisera tested showed any inhibitory effect on oxygen evolution with untreated inside-out thylakoids. Thus the antibodies did not interact with any active sites of the proteins.

The amount of the 23 and 16 kDa proteins were determined by rocket immunoelectrophoresis in the membranes of unfractionated thylakoids, inside-out thylakoids (derived from the appressed regions (30) and stroma lamellae vesicles. This technique which allows the quantification of a small amount of a certain protein species in a complex protein mixture has previously been used in studies on thylakoid proteins such as CF_1 , ferredoxin, reductase and plastocyanin (31-33). Our results showed that unfractionated thylakoids contained one 23 kDa protein per some 170 chlorophylls. The value for the 16 kDa protein was one per 205 chlorophylls. Previously, the 33 kDa protein was shown to be present in one copy per 200 chlorophylls (B. Andersson et al., unpublished observation). Fractionation of the thylakoids revealed that both the 23 and 16 kDa proteins were highly enriched in the appressed regions, as judged by their content in highly purified inside-out vesicles as compared to stroma lamellae vesicles. On a chlorophyll basis there was 6-8 times more of these proteins in appressed regions as compared to non-appressed regions.

Control IgG

Anti-23

Anti-16

Inside-out
thylakoidsRight-side out
thylakoids

Fig.2. Immunoprecipitation of inside-out and right-side out thylakoid vesicles. IgG from antisera against the 23 and 16 kDa proteins, and from non-immune serum were used. The final concentration in 20 μ l were 2mg IgG/ml and 50 μ g chl/ml.

Washing inside-out vesicles with increasing concentrations of NaCl (10-300 mM) resulted in a increasing deactivation of oxygen evolution (Fig. 4). Using rocket immunoelectrophoresis the amounts of the 33, 23 and 16 kDa proteins remaining on the membrane after washing were quantified. Fig. 3 shows an example of the rockets obtained for the 23 kDa protein remaining in inside-out vesicles after washing with increasing concentrations of salt. The area of these rockets was determined and is depicted in the diagram of Fig. 3. The same procedure was used for the 16 and 33 kDa proteins. Based upon such immunoelectrophoresis data we have correlated the release of these

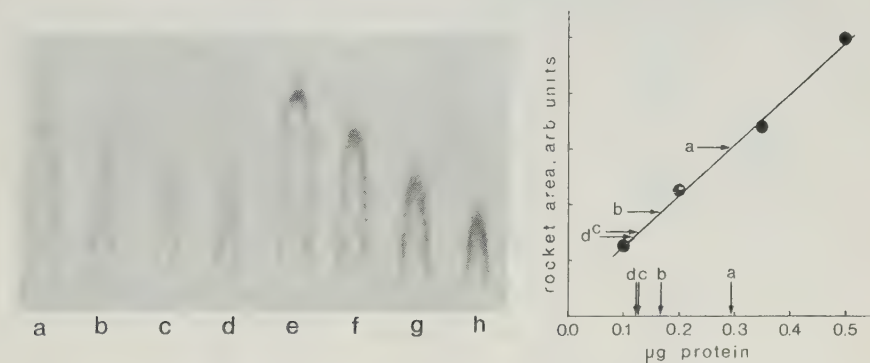


Fig. 3. Rocket immunoelectrophoresis, using the antiserum against the 23 kDa protein. Samples were; (a) untreated inside-out thylakoids corresponding to 1.8 µg chlorophyll, (b-d) inside-out thylakoids washed with 100, 125, and 150 mM NaCl respectively, (e-h) 0.50, 0.35, 0.20, and 0.10 µg of the purified 23 kDa protein respectively. The diagram shows the corresponding calibration curve and the quantification of the thylakoid samples.

proteins with the inhibition of oxygen evolution (Fig. 4). Strikingly, the release of the 23 kDa protein followed very closely the degree of inhibition. In contrast, the release of the 16 kDa protein markedly exceeded the inhibition; at 30% inhibition as much as 75% of the protein was released. Usually not more than 80% of the 23 and 16 kDa proteins could be

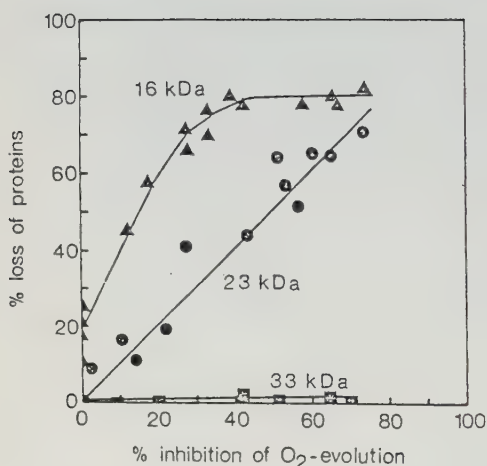


Fig. 4. Correlation between the release of the 33, 23, and 16 kDa-proteins and the inhibition of oxygen evolution after washing inside-out thylakoids with increasing concentrations of NaCl.

released, probably due to the approximately 20% contamination by right-side out vesicles (34). Virtually no 33 kDa protein was released by the salt washings. Moreover, salt-washing did not release any manganese (17). This is in contrast to alkaline Tris treatment which released all three proteins (Fig. 5) and which is known to also release manganese.

In order to investigate the molecular organization of the 33, 23 and 16 kDa proteins on the inner thylakoid surface rebinding of the three proteins to either salt or Tris-washed inside-out vesicles was performed (Fig. 5). Usually a 5-10 times excess of each protein (based on the stoichiometry in untreated material) was added to depleted inside-out vesicles under low ionic strength. Upon readdition of the 23 kDa protein to salt-washed inside-out vesicles it was rebound to 93% of the original level. Readdition of the 16 kDa protein resulted only in a partial rebinding. However, if the 16 kDa protein was added after the 23 kDa protein was rebound, the binding of the 16 kDa protein was markedly enhanced reaching nearly 90% of the original level.

This indicates that the 23 kDa protein is involved in the binding of the 16 kDa protein. When the 33, 23 and 16 kDa proteins were added individually to the Tris washed vesicles only the 33 kDa protein showed any significant rebinding. A partial rebinding of the 23 kDa protein to the Tris washed vesicles was obtained after the 33 kDa protein had been rebound. In contrast, the presence of the 33 kDa protein did not enhance the rebinding of the 16 kDa protein. Thus the 33 kDa protein seems necessary for the binding of the 23 kDa protein but not directly for the 16 kDa protein.

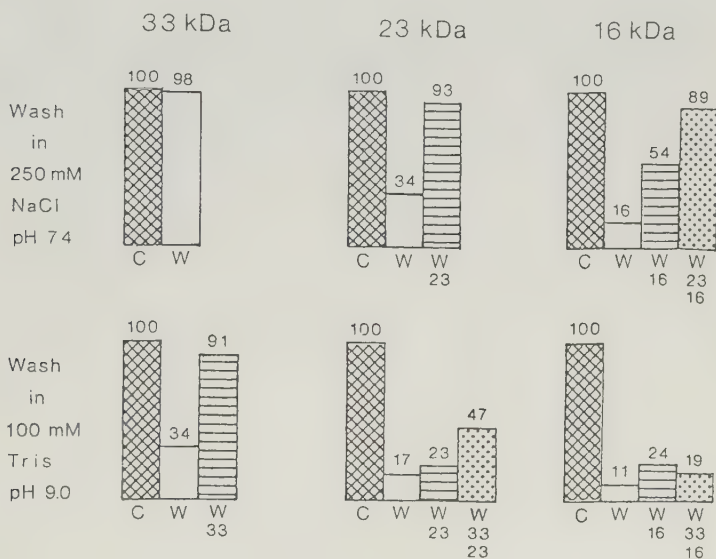


Fig. 5. Determination of protein rebinding to salt washed or Tris washed inside-out vesicles by rocket immunoelectrophoresis. The level of each protein (33, 23 and 16 kDa) in untreated vesicles (C) was set to 100% to be compared with the levels in washed vesicles (W). For rebinding each of the three proteins (20 μ g) was added individually to washed vesicles (100 μ g Chl.) suspended in 5 ml of 70 mM sucrose, 30 mM sodium phosphate pH 6.5, 3 mM NaCl. After incubation for 30 min. the vesicles were sedimented before being analysed. When more than one protein was added, the vesicles were sedimented and resuspended before the second protein was added to achieve a sequential rebinding.

DISCUSSION

Quantification of the 33, 23, and 16 kDa proteins by rocket immunoelectrophoresis showed that there is approximately one copy of each polypeptide per 200 chlorophylls in intact spinach thylakoids. The stoichiometry between these proteins and the photosystem 2 reaction centre depends on which chlorophyll/P680 or chlorophyll/Q ratio applies to spinach thylakoids. Using repetitive short flashes a chlorophyll/Q ratio of 550 was obtained (35). Using another spectrophotometric method, Melis and Harvey reported a chlorophyll/Q ratio of 250-300 (36). The first ratio results in 2-3 molecules of each polypeptide per photosystem 2 reaction centre while the latter ratio gives a value of 1-2. Moreover, the 23 and 16 kDa proteins were highly enriched in subfractions originating from the appressed thylakoid region, demonstrating their association with photosystem 2. Although quite depleted in stroma lamellae vesicles, reproducible amounts of the proteins could always be detected. This may suggest that photosystem 2 in non-appressed thylakoids; possibly photosystem 2 β (37), also contains the 33, 23 and 16 kDa proteins.

The agglutination studies show that these proteins are exposed on the inner side of the thylakoid membrane. Furthermore, the conditions needed to release the proteins (2,5), and their solubility in water without the use of detergents (17,18) suggest that they are extrinsic proteins located at the inner thylakoid surface. The fact that these proteins show pronounced enrichment in the appressed thylakoid regions is quite interesting. Previously, such an extreme lateral heterogeneity between the appressed and non-appressed thylakoids has only been demonstrated for proteins exposed at the outer membrane surface (38). For those, a lateral displacement may be explained by the influence of attractive and repulsive forces in the tight appressions (39,40). The 33, 23 and 16 kDa proteins probably belong to the photosystem 2 membrane spanning complex able to sense the outside environment. This is supported by a preliminary immunoprecipitation study (41). This showed that when added to a

partly solubilized photosystem 2 preparation (42) , antibodies against the 33 and 23 kDa proteins did not only precipitate the antigenic proteins but also proteins of 47, 43, 27, 22, and 10 kDa, where at least the 47 and 43 kDa proteins are spanning the membrane (43) . Additionally, attractive and repulsive forces on the luminal side of the thylakoid (44) could also contribute to the lateral heterogeneity of the 33, 23, and 16 kDa proteins.

The rebinding studies showed that the 33 kDa protein could rebind independently of the 23 and 16 kDa proteins suggesting that it is bound to some intrinsic membrane protein. The 23 kDa protein appears to bind to the 33 kDa protein. Probably, the 23 kDa protein also has a binding site on an intrinsic membrane protein, since it can be retained on the membrane when the 33 kDa protein is released by mild Tris treatment (Andersson et al., unpublished observations) or urea treatment (45) . Finally, the 16 kDa protein appears to be bound to the 23 kDa protein. A similar rebinding pattern for these three proteins has recently been demonstrated from studies on photosystem 2 particles (8,45) . Thus, the three proteins seem to be arranged in one complex at the inner thylakoid surface and in probable association with intrinsic photosystem 2 proteins. Both the 23 and 16 kDa proteins are likely to be bound by electrostatic forces since their release and rebinding is dependent on the ionic environment. In contrast, the 33 kDa protein was not released by high salt (Fig. 5) indicating that other than electrostatic forces may be involved in its binding to the membrane. A hydrophobic binding is not likely since the protein is released by increasing the temperature to 53°C (L.E. Andreasson, personal communication) and hydrophobic interactions are strengthened at higher temperatures (46) . Rather, hydrogen bonding may be involved in the attachment of the 33 kDa protein, in accordance with its release at high pH or increasing temperature.

The strict correlation between the release of the 23 kDa protein and the inactivation of the oxygen evolving activity (Fig. 4) after salt-washing suggest an obligatory role for this protein in photosynthetic water oxidation. Furthermore, if the protein is an oligomer, as may be suggested from its

stoichiometry in relation to the photosystem 2 reaction centre, it is also released in that form. An obligatory role for the 23 kDa protein has also been supported by recent kinetic studies (47,48)

Very recent results (49) show that the 23 kDa protein increases the affinity of the water oxidation site for chloride. This indicates that the obligatory role for this protein in oxygen evolution may apply only under low chloride conditions. This may explain discrepancies in the assignment of the 23 kDa protein as obligatory (Fig. 4, (47,48)) or supplementary (4,8) for oxygen evolution.

The release pattern of the 16 kDa protein (Fig. 4) argues against an obligatory role in oxygen evolution. However, considering that the protein may be a dimer, a release curve close to that observed would be obtained if the subunits were released independently and only one was necessary for the activity.

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REFERENCES

1. Åkerlund, H.-E. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.) vol. 2, pp 465, Balaban International Science Service, Philadelphia, PA
2. Åkerlund, H.-E. and Jansson, C. (1981) FEBS Lett. 124, 229-232
3. Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) FEBS Lett. 133, 265-268
4. Kuwabara, T. and Murata, N. (1983) Plant and Cell Physiol. 24, 741-747
5. Åkerlund, H.-E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1-10.
6. Ljungberg, U., Jansson, C., Andersson, B. and Åkerlund, H.-E. (1983) Biochem. Res. Commun. 113, 738-744
7. Lindberg-Møller, B.L. and Hoj, P.B. (1983) Carlsberg Res. Commun. 48, 161-185
8. Miyao, M. and Murata, N. (1983) Biochim. Biophys. Acta 725, 87-93
9. Fukutaka, E., Imaoka, A., Akabori, K. and Toyoshima, Y. (1983) FEBS Lett. 158, 217-221
10. Cammarata, K., Tamura, N., Sayre, R. and Cheniae, G. (1984) in Proceedings of the 6th International Congress on Photosynthesis. (Sybesma, C., ed.) Martinus Nijhoff, The Hague, in press
11. Metz, J.G., Wong, J. and Bishop, N.I. (1980) FEBS Lett. 114, 61-66
12. Metz, J. and Bishop, N.I. (1980) Biochem. Res. Commun. 90, 560-566
13. Bricker, T.M., Metz, J.G., Miles, D. and Sherman, L.A. (1983) Biochim. Biophys. Acta 724, 447-455
14. Dismukes, G.C., Abromowics, D.A., Ferris, K.F., Mathur, P., Siderer, Y., Upadrashta, B. and Watnick, P. (1984) in Proceedings of the 6th International Congress on Photosynthesis (Sybesma, C., ed.) Martinus Nijhoff, The Hague, in press

15. Yamamoto, Y., Shimada, S. and Nishimura, M. (1982) FEBS Lett. 151, 49-53
16. Kuwabara, T. and Murata, N. (1983) in Oxygen-evolving System of Photosynthesis (Inoue, Y., Crofts, A.E., Govindjee, Murata, N., Renger, G. and Satoh, K. eds.) pp 223-227, Academic Press, Tokyo
17. Jansson, C., Åkerlund, H.-E. and Andersson, B. (1983) Photosynth. Res. 4, 271-279
18. Jansson, C. (1984) in Proceedings of the 6th International Congress on Photosynthesis. (Sybesma, C., ed.) Martinus Nijhoff, The Hague, in press
19. Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 591-599
20. Satoh, N., Nakatani, H.Y., Steinback, N.E., Watson, J. and Arntzen, C.J. (1983) Biochim. Biophys. Acta 724, 142-150
21. Pfister, N., Steinback, N.E., Gardner, G. and Arntzen, C.J. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 981-985
22. Burke, J.J., Ditto, C.L. and Arntzen, C.J. (1979) Arch. Biochem. Biophys. 187, 252-263
23. Haworth, P., Watson, J.L., and Arntzen, C.J. (1983) Biochim. Biophys. Acta 724, 151-158
24. Bittner, M., Kupferer, P. and Morris, C.F. (1980) Anal. Biochem. 103, 459-471
25. Laurell, C.-B. (1969) Anal. Biochem. 15, 45-52
26. Åkerlund, H.-E. and Andersson, B. (1983) Biochim. Biophys. Acta 725, 34-40
27. Andersson, B. (1984) in Proceedings of the 6th International Congress on Photosynthesis (Sybesma, C., ed) Martinus Nijhoff, The Hague, in press
28. Arnon, D.I. (1949) (1949) Plant Physiol. 24, 1-15
29. Bearden, J.C. Jr. (1978) Biochim. Biophys. Acta 533, 525-529

30. Andersson, B., Sundby, C. and Albertsson, P.-Å. (1980) *Biochim. Biophys. Acta* 599, 391-402
31. Böhme, H., (1978) *Eur. J. Biochem.* 83, 137-141
32. Haehnel, W., Berzborn, R.J. and Andersson, B. (1981) *Biochim. Biophys. Acta* 637, 389-399.
33. Berzborn, R.J., Müller, D., Roos, P. and Andersson, B. (1981) in *Proceedings of the 5th International Congress on Photosynthesis* (Akoyunoglou, G., ed.), vol. 3, pp 107-120, Balaban International Science Services, Philadelphia, PA
34. Andersson, B., Simpson, D.J. and Hoyer-Hansen, G. (1978) *Carlsberg Res. Commun.* 43, 77-89
35. Andersson, B. and Haehnel, W. (1982) *FEBS Lett.* 146, 13-17
36. Melis, A. and Harvey, G.W. (1981) *Biochim. Biophys. Acta* 637, 138-145
37. Anderson, J.M. and Melis, A. (1983) *Proc. Natl. Acad. Sci. U.S.A.* 80, 745-749
38. Anderson, J.M. and Andersson, B. (1982) *Trends Biochem. Sci.* 7, 288-292
39. Barber, J. (1980) *FEBS Lett.* 118, 1-10
40. Barber, J. (1982) *Annu. Rev. Plant Physiol.* 33, 261-295
41. Ljungberg, U., Jansson, C., Larsson, C., Åkerlund, H.-E. and Andersson, B. (1984) in *Proceedings of the 6th International Congress on Photosynthesis*. (Sybesma C., ed.) Martinus Nijhoff, The Hague, in press
42. Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) *FEBS Lett.* 134, 231-234
43. Andersson, B., Anderson, J.M. and Ryrie, I.J. (1982) *Eur. J. Biochem.* 123, 465-472
44. Albertsson, P.-Å (1982) *FEBS Lett.* 149, 186-190

45. Murata, N., Miyao, M. and Kuwabara, T. (1983) in *The Oxygen-evolving System of Photosynthesis* (Inoue, Y., Crofts, A.E., Govindjee, Murata, N., Renger, G. and Satoh, K. eds) pp 213-222, Academic Press, Tokyo
46. Tanford, C. (1980) in *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, 2nd ed., John Wiley and Sons, New York
47. Åkerlund, H.-E., Renger, G., Weiss, W. and Hagemann, R. (1984) *Biochim. Biophys. Acta*, in press
48. Åkerlund, H.-E., Brettel, K. and Witt, H.T. (1984) *Biochim. Biophys. Acta*, in press
49. Andersson, B., Critchley, C., Ryrrie, I.J., Jansson, C., Larsson, C., and Anderson, J.M. (1984) *FEBS Lett.*, in press

